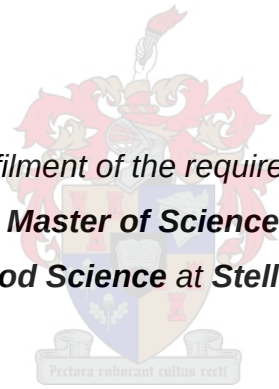
Identifying potentially valuable flavour fractions from South African botanical extracts using LC Taste® as a rapid screening method

by

Emma van der Merwe



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Supervisor: Prof. Gunnar Oliver Sigge

Co-supervisor: Prof. André Joubert de Villiers

2nd Co-supervisor: Dr Martin Dovey

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DECLARATION

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Emma van der Merwe
December 2020

ABSTRACT

The enormous variety of southern African botanicals offers unending opportunities for food and beverage applications. Considering the growing demand for botanical products and preparations as natural sources of flavour and/or functionality, the leaves of the botanical species, *Moringa oleifera* (Moringa), *Warburgia salutaris* (Pepperbark) and *Cyclopia genistoides* (Honeybush) and the fruit pulp of *Adansonia digitata* (Baobab) were analysed for potentially valuable flavours using the flavour screening technique, LC Taste® (Liquid Chromatography Taste®). Concentrated extracts of the preferred flavour from each botanical were prepared using exploratory steps. The concentration of ethanol (0%, 50%, 100%, $v.v^{-1}$) in the aqueous solvent, the solid-liquid ratio (15%, 25%, 50%, $w.v^{-1}$), the effect of sonication versus maceration with magnetic stirring, and the effect of the duration of the extraction time (3 h, 6 h, 24 h) at elevated temperature (70°C) on extraction efficiency were explored. The flavour extraction efficiency was evaluated based on sensory evaluation as well as relative peak intensities of the high performance liquid chromatography-diode-array detection (HPLC-DAD) signals. The preferred flavour was extracted from each botanical using 50% ethanol ($v.v^{-1}$). Although the maximum solid-liquid ratio produced concentrated extracts, the ratio should ideally be further optimised per botanical. Maceration resulted in better extraction of flavour from Honeybush, Moringa and Baobab, while sonication extracted more flavour from Pepperbark. While 24 h extracted more flavour from Honeybush, the taste- and peak intensities of Pepperbark, Baobab, and Moringa, decreased with increasing extraction time at elevated temperature (70°C), suggesting that the compounds responsible for their flavour are relatively susceptible to thermal degradation. Extracts of each botanical was analysed via LC Taste®, optimising the HPLC parameters per botanical. One-minute fractions were collected in established fraction collection windows. The fractions were tested for the presence of *Salmonella* spp. based on International Organization for Standardization (ISO) 6579: - 1:2017 method, and for *Bacillus cereus* spores using an adapted version of the ISO 7932:2004 method. The fraction that gained the interest of the tasting panel was a pungent fraction from Pepperbark. To identify and quantify the compound(s) responsible for the characteristic heat, further chromatography was performed. The gas chromatography-mass spectrometry (GC-MS) results suggested that few aromatic compounds were isolated in the fraction. Liquid chromatography-electrospray ionisation–mass spectrometry (LC-ESI-MS) and tandem mass spectrometry (MS/MS) enabled the identification of the most abundant compound in the fraction, the pungent piperine-type alkaloid, piperanine. The current study marks the first report of piperanine in *W. salutaris* as well as in the genus *Warburgia.* Piperanine was extracted from the Pepperbark sample using ultrasound-assisted extraction (UAE) at 50°C with a solid-solvent ratio of 1:10, while a variety of solvents (100% ethanol and 50% aqueous ethanol ($v.v^{-1}$); acidified aqueous solvents; pH 1.00, pH 4.00; pH 7.00) and

extraction times (18 min and 3 h) were applied. The maximum yield, extracted in 3 h, using 50% aqueous ethanol, suggested that 0.412% ($w.w^{-1}$) of the dehydrated leaves constitutes piperanine. This quantity is subject to natural variation and further investigation could improve extraction yields. Although piperanine was the likely sole contributor of pungency detected in the fraction isolated from the *W. salutaris* extract, a variety of other hot-tasting compounds, sesquiterpenes, have been associated with the *Warburgia* species. It is suspected that the initial extraction conditions of 50% aqueous ethanol ($v.v^{-1}$) excluded the extraction of these compounds. Extracts of *W. salutaris* leaves with enhanced extraction yields of piperanine and pungent sesquiterpenoids, hold great potential for culinary applications as a novel flavour source with potential functionality.

UITTREKSEL

Die suider Afrika plantdiversiteit bied oneindige geleenthede vir die gebruik in die voedselbedryf. Om die toenemende aanvraag vir botaniese bestandele as natuurlike bronne van geur en/of funksionaliteit aan te spreek, is die blare van die botaniese spesies, *Moringa oleifera* (Moringa), *Warburgia salutaris* (Peperbasboom) en *Cyclopia genistoides* (Heuningbos), asook die vrugte pulp van *Adansonia digitata* (Kremetart), geanaliseer met die geurkeuringstegniek, 'LC Taste®' ('Liquid Chromatography Taste®'), om potensieel waardevolle geure te identifiseer. Eksperimentele stappe is gevolg om gekonsentreerde ekstrakte van die voorkeurgeur van elke spesie voor te berei. Die konsentrasie van etanol (0%, 50%, 100%, $v.v^{-1}$) in die oplosmiddel, the verhouding van die plantmateriaal tot die oplosmiddel (15%, 25%, 50%, $w.v^{-1}$), die effek van ultrasoniesebehandeling in vergelyking met maserasie met 'n magnetisie roerder, en die effek van die die ekstraksietyd (3 h, 6 h, 24 h) by verhoogde temperatuur (70°C) op die ekstraksiedoeltreffendheid was bestudeer. Die ekstraksiedoeltreffendheid was geëvalueer gebaseer op sensoriese analises en op piekintensiteit van 'hoë druk vloeistof chromatografie-diode-array detection' (HPLC-DAD) seine. Die verkeurgeur was ge-ekstraheer van elke spesie met die 50% etanol ($v.v^{-1}$). Alhoewel die maksimum plantmateriaal-oplosmiddel verhouding gekonsentreerde ekstrakte geproduseer het, moet die verhouding verkieslik geoptimiseer word per plantspesie. Met betrekking tot die ekstraksiemetodes, het maserasie tot beter ekstraksie gelei in die geval van Heuningbos, Moringa en Kremetart, terwyl ultrasoniese behandeling meer geur uit Peperbasboom ge-ekstraheer het. Terwyl 24 h meer geur ge-ekstraheer het uit Heuningbos, het die pieke- en die smaakintensiteit van Peperbasboom, Kremetart, en Moringa gedaal met verlengde tyd by verhoogde temperatuur (70°C). Dit suggereer dat die komponente verantwoordelik vir hul geur relatief sensitief is tot termiese afbreking. Gekonsentreerde ekstrakte van elke plantspesie is deur 'LC Taste®' gefraksioneer. Een-minuut fraksies is in die gevestigde fraksie-kolleksie tydperk gekollekteer en getoets vir die teenwoordigheid van *Salmonella* spp. gebaseer op die Internasionale Organisasie van Standaardisering (ISO) 6579: - 1:2017 metode, en vir *Bacillus cereus* spore met 'n aangepaste weergawe van die ISO 7932:2004 metode om hul mikrobiese veiligheid te bewys voordat hulle deur 'n sensoriese paneel geproe is. Die fraksie wat die paneel die meeste geïntriseer het was 'n pikante fraksie van die Peperbasboom. Die gas chromatografie–massa spektromatrie (GC-MS) resultate van die fraksie het gedui dat min aromatiese komponente in die fraksie geïsoleer is. Vloeistof chromatografie–elektrosproei ionisasie–massa spektrometrie (LC-ESI-MS) en tandem massa spektrometrie (MS/MS) analises het gelei tot die identifisering van die volopste verbinding in die fraksie, die pikante piperine-analoog, piperanine. Die huidige navorsing is die eerste verslag van piperanine in *W. salutaris*, en die genus, *Warburgia*. Ultrasoniesebehandeling (UAE) by 50°C met 'n plantmateriaal-oplosmiddel verhouding van 1:10, is toegapas om

piperanine te ekstraheer, asook verskillende oplosmiddels (100% etanol en 50% etanol ($v.v^{-1}$); pH-geadapteerde water; pH 1.00, pH 4.00; pH 7.00) en ekstraksietye (18 min en 3 h). Die maksimum opbrengs, ge-ekstraheer in 3 h met 50% etanol ($v.v^{-1}$), dui dat 0.412% ($w.w^{-1}$) van die gedroogte blare bestaan uit piperanine. Hierdie waarde is geneig tot natuurlike variasie en verdere navorsing kan die opbrengs verbeter. Alhoewel piperanine die enigste bydraer is tot die pikante smaak in die geïsoleerde fraksie, is ‘n reeks ander brandwarm komponente, geklassifiseer as sesquiterpenoÏedes, vantevore met die *Warburgia* spesie geassosieer. Die aanvanklike ekstraksie kondisie van 50% etanol ($v.v^{-1}$) het moontlik die minder polêre komponente uitgesluit. Ekstrakte van *W. salutaris* blare met verhoogde konsentrasies van piperanine en die pikante sesquiterpenoÏedes, hou potensiaal vir voedselgebruik as ‘n onbekende bron van geur en die medisinale eienskappe.

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NOTES

This thesis is presented in the format prescribed by the Department of Food Science, Stellenbosch University. The structure is in the form of two research chapters and is prefaced by an introduction chapter with the study objectives, followed by a literature review chapter and culminating with a general discussion and conclusions. Language, style and referencing format used are in accordance with the requirements of the *International Journal of Food Science and Technology.* This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between the chapters has, therefore, been unavoidable.

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CHAPTER 1
GENERAL INTRODUCTION

In recent years, there has been an increased demand for the use of botanical products and preparations in the food and beverage sector (Harnly *et al.,* 2017; Mantzourani *et al.*, 2019; Nazir *et al*., 2019). Their natural colour, flavour and associated health benefits lend themselves excellently to addressing the modern consumer's desire for wholesome alternatives to synthetic ingredients (Gruenwald, 2009; Perestrelo *et al*., 2017; Mantzourani *et al.*, 2019; Nazir *et al.*, 2019). This trend is fueled by growing consumer awareness and science-based evidence of the health properties of botanical ingredients (Mantzourani *et al.*, 2019; Nazir *et al.*, 2019). Medicinal plants have also made their way into food and beverage products, offering interesting new tastes and flavours in addition to potential health-enhancing effects (Gruenwald, 2009; Van Wyk, 2011; Perestrelo *et al*., 2017; Nazir *et al.,* 2019). Even if concentrations of bioactive components are too low for any actual pharmacological claims, consumers are attracted to the natural ingredients as well as their associated benefits (Gruenwald, 2009; Nazir *et al*., 2019).

Of course, the enormous variety of South African botanicals provides unending opportunities to find new flavours or tastes with potential functionality (Van Wyk, 2011; Masondo & Makunga, 2019). A few examples of edible South African plant species, sourced from indigenous plants, that have already been commercialised, includes gum Arabic, baobab, buchu, *waterblommetjies*, rooibos tea, honeybush tea, finger millet, sour fig, jelly melon, palm wine, pearl millet, Livingston potato, marula fruit, sorghum, jugo bean and cowpea (Van Wyk, 2011).

An article published by Van Wyk (2011), reports on a list of more than 120 indigenous South African species with culinary significance and comments on their potential application in food products.

Among the host of botanical ingredients available for culinary application, many have been around for a long time while other less familiar ingredients have only recently been discovered by the food industry (Gruenwald, 2009; De Vynck *et al.*, 2016). The full potential of various botanicals has not been realised yet, creating opportunities to discover novel flavours and tastes with potential for food and beverage application (Harnly *et al.,* 2017). There is room for applications of known raw materials by creating new applications or taste profiles or by scientific elucidation of previously unknown functions (Ramos *et al*., 2019). New discoveries and extractions will pave the way to developing new products.

With the goal in mind of rapidly screening complex food matrices for potentially valuable or off-flavours, the novel flavour screening technique, LC Taste®, was developed (Reichelt *et al*., 2010a). LC (Liquid Chromatography) Taste® is an analytical method that combines the separation or fractionation of non-volatiles from a food matrix using reverse

phase-high temperature liquid chromatography (RP-HTLC) with sensory analysis (Reichelt *et al*., 2010a). A blend of non-toxic solvents is used, enabling sensory evaluation by online or direct sensory evaluation, without the need to remove harmful solvents from the collected fractions (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c). Despite the convenience and time-saving aspects offered by LC Taste®, chemical constituents are protected against deterioration and chemical changes that often occur as a consequence of complicated isolation and purification steps required after conventional high-performance liquid chromatography (HPLC) fractionation using toxic eluents (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c).

Many liquid chromatographic methods can be used for the isolation of non-volatile compounds from complex natural products, for example preparative HPLC (pHPLC), or liquid-liquid partition chromatography techniques, such as high speed counter-current chromatography (HSCCC) or fast centrifugal partition chromatography (FCPC) (Reichelt *et al*., 2010b). These methods require toxic solvents and can therefore not be directly used for human taste evaluations. Cost-, labour- and time-intensive procedures are required for the isolation of single compounds or interesting fractions before sensory testing becomes safe for the tasting panel (Reichelt *et al*., 2010b; Yabré *et al*., 2018). LC Taste® was designed provide a more rapid and efficient alternative and to overcome the limitations of conventional separations of food mixtures (Reichelt *et al*., 2010a). With LC Taste®, high performance liquid chromatography could, for the first time, be used for the separation of aroma and flavouring substances from solutions while performing simultaneous sensory evaluations (Symrise, 2005).

This novel technique enables the correlation of sensory data to analytical detection, for example, liquid chromatography–mass spectrometry (LC-MS) or liquid chromatography–diode array detection (LC-DAD), allowing structural elucidation and quantification, if required (Reichelt *et al.,* 2010b; Mittermeier *et al*., 2018).

To produce concentrated botanical extracts to be subjected to LC Taste®, factors including the plant part, its physicochemical properties and tissue matrix type are all factors that need to be considered (Azmir *et al*., 2013; Belwal *et al*., 2018). The cell structure, form of target compounds (bound or free), moisture content, and particle size are some of the most important properties of a botanical ingredient to be extracted (Pronyk & Mazza, 2009; Belwal *et al*., 2018; Anbalagan *et al.*, 2019; Zhang *et al.*, 2019).

The solvent, extraction time and temperature, and the solid-liquid ratio should be carefully selected since their effect on the nature and the yield of the secondary metabolites extracted from the base plant material is critical (Chan *et al*., 2014; Nastic *et al.,* 2018; Anbalagan *et al.*, 2019; Dirar *et al.*, 2019; Zhang *et al*., 2019).

Conventional techniques to extract bioactive compounds from botanical sources are generally based on the extracting power of different solvents used in combination with the effect of heat and/or mixing (Azmir *et al*., 2013). Typically used, classical extraction techniques used to extract bioactive compounds from vegetative materials include Soxhlet extraction, maceration and hydrodistillation (Azmir *et al*., 2013). To accommodate the growing demand of herbal products for wider and safer applications, there has been increased effort to provide high-quality botanical extracts with improved yields and lower production costs (Belwal *et al*., 2018). As a result, extraction techniques that have been developed are known for their reduced extraction time and volume of organic and toxic solvents, their simplicity and enhanced extraction yields with lower energy consumption, making them more environmentally friendly (Belwal *et al*., 2018). Examples of these techniques are microwave assisted extraction (MAE), supercritical fluid extraction (SFE), pressurised liquid extraction (PLE), ultrasonic assisted extraction (UAE), pulsed electric field assisted extraction (PEF) and enzyme assisted extraction (EAE) (Belwal et al., 2018).

To this day, there has been no reported work on the application of LC Taste® to screen flavour fractions from extracts of *Adansonia digitata* (Baobab) dried fruit pulp, *Moringa oleifera* (Moringa) dried leaf powder, *Cyclopia genistoides* (Honeybush) fermented tea nor from *Warburgia salutaris* (Pepperbark) dried leaf powder.

Considering the exciting opportunities available for product development using botanical ingredients, this study aims to identify potentially valuable flavours and/or tastes from these South African botanicals by applying LC Taste® as a rapid screening method.

With this research aim in mind, the objectives of this study are to manipulate the extraction conditions for each of the botanicals to produce concentrated extracts of the preferred flavour profile from each botanical source. The LC TasteC protocol will be optimised for each botanical, using sensory evaluation to identify eluted flavour fractions of interest. The eluted flavour fractions will be subjected to microbial testing to ensure their safety before tasting. The fractions of interest, as identified via LC Taste®, will be subjected to further chromatography with the goal of identifying the specific compound(s) responsible for the perceived flavour.

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CHAPTER 2
LITERATURE REVIEW

1. INTRODUCTION

Extracts from traditional South African botanicals offer endless flavours as well as nutritional advantages to use in in the food industry (Gruenwald, 2009). These extracts are ideal to fill the gap in the growing market for natural food additives (Gruenwald, 2009; Perestrelo *et al*., 2017; Mantzourani *et al.*, 2019; Nazir *et al.*, 2019).

Increased consumer awareness to health and nutrition has led towards the movement from synthetic ingredients to natural alternatives (Mantzourani *et al.*, 2019; Nazir *et al.*, 2019). Superfoods and other beneficial compounds, including a variety of minerals and vitamins, antioxidants and fatty acids have been successfully applied to beverages to create new functional beverages, juices, fortified water, tea and dairy products (Gruenwald, 2009; Mantzourani *et al.*, 2019; Nazir *et al.*, 2019). These drinks have grown in popularity, convenience, creativity and taste while maintaining their healthy status (Gruenwald, 2009; Mantzourani *et al.*, 2019; Nazir *et al.*, 2019).

South African foods and flavours form the foundation of many successful industries, such as Amarula® cream liqueur, rooibos tea, sorghum beers and multiple breakfast cereals, to highlight a few (Van Wyk, 2011). With novelty being a driving factor in today's market, the rich diversity of South African botanicals provides undeniable opportunities for product development, including new foods, flavours and beverages that embody the cultural abundance of South Africa (Van Wyk, 2011). The full potential of many botanicals has not been realised, leaving room for the discovery of exciting new flavours or by the improvement of known plant extracts (De Vynck *et al.*, 2016; Harnly *et al.,* 2017; Ramos *et al*., 2019).

LC (Liquid Chromatography) Taste® is a novel analytical method that provides a rapid method for the flavour screening of complex mixtures, as in the case of botanical extracts, to identify taste-active compounds (Reichelt *et al*., 2010a). The protocol combines the fractionation of a multi-component food matrix using high temperature liquid chromatography (HTLC) with sensory analysis, enabling the relation of analytical to sensory data (Reichelt *et al*., 2010a).

In this study, the LC Taste® protocol is applied to explore the potential of a range of South African botanicals, namely Baobab (*Adansonia digitata*), Moringa (*Moringa oleifera*), Honeybush (*Cyclopia genistoides*) and Pepperbark (*Warburgia salutaris*) as flavour sources for the food and beverage industry.

2. SOUTH AFRICAN BOTANICALS

2.1 The diversity of South African flora

South Africa is home to an abundance of biodiversity, unequalled by other temperate regions, earning a place in the top 25 most biodiverse nations (Reyers *et al*., 2001). In addition, South Africa boasts the fifth highest number of plant species in the world (Cowling *et al.*, 1997; Reyers *et al*., 2001).

The incredibly diverse fynbos ecosystem in South Africa, located at the south-western tip (Figure 2.1), is renowned for being the world's richest site of plant biodiversity and endemism (Cowling *et al.*, 1997). Recognised as the smallest, yet most diverse of the six floral kingdoms of the world, the Cape Floristic Region, supports the growth of around 9 000 plant species of which 70% are endemic (Soderberg & Compton, 2007). The dominating form of vegetation in this 90 000 km^2 zone is fynbos - a hardy, fire-prone, hard-leafed shrubland that grows only in SA (Cowling *et al*., 1997; Soderberg & Compton, 2007). This form of vegetation thrives on nutrient-poor, sandy soils, common to quartzitic mountains and windblown sands of the outer coastal region of the lowlands (Cowling *et al*., 1997). They have adapted to long periods of drought and periodic fires (Soderberg & Compton, 2007).

Despite their spectacular beauty, fynbos ecosystems are of great value to the South African economy as they deliver an array of services to society, providing consumptive and non-consumptive uses (Cowling *et al*., 1997). These include ecotourism opportunities, water supply and a large biodiversity depot, providing many potentially valuable horticultural, food and drug sources (Cowling *et al*., 1997).

A few fynbos plants have been commercialised as food and drug products. The best known is rooibos tea, which is enjoyed in more than 37 countries globally (Joubert & De Beer, 2011).

The wealth of South African vegetation is, however, supported by its entire range of biomes, including fynbos, Nama-Karoo, savannah, Succulent Karoo, grassland, forest, Albany Thicket, Indian Ocean Coastal belt and desert biomes (Potts *et al.*, 2015). The figure below illustrates the distribution of the various South African biomes, each supporting unique plant growth (Figure 2.1).

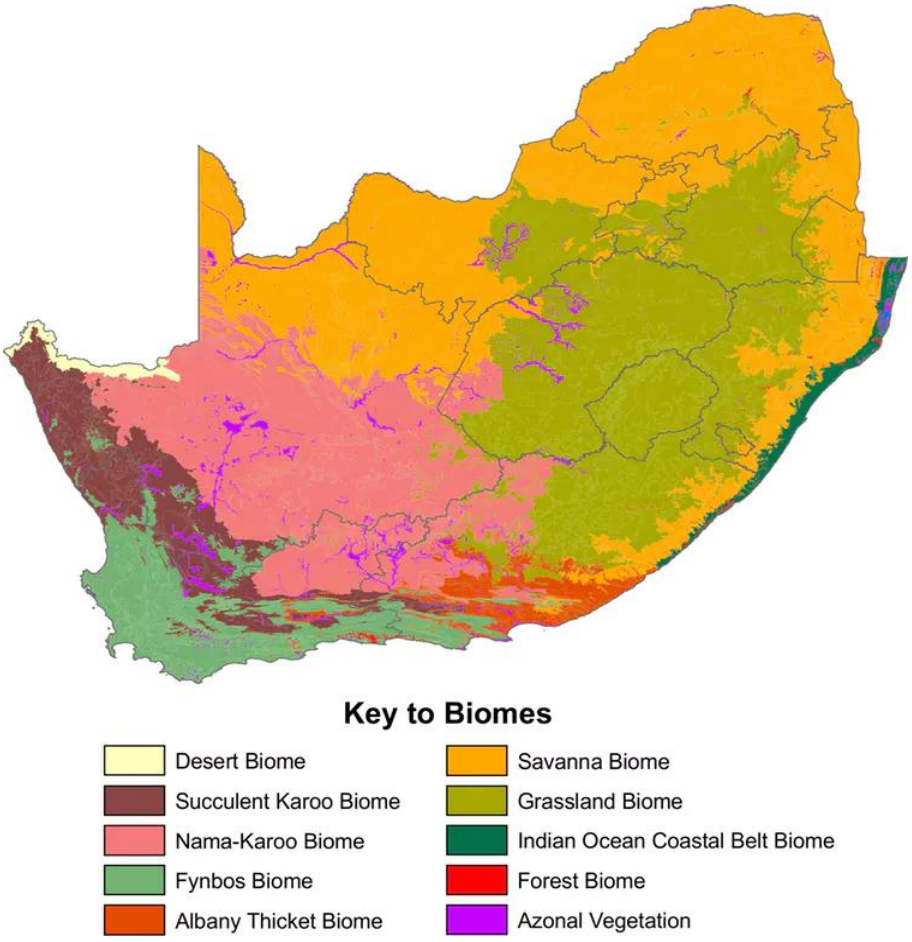

Figure 2.1 The distribution of the nine South African biomes (Huntley, 2016)

2.2 Traditional and current use as well as the future potential of South African plants in the food industry

By 2005, 119 of the enormous range of edible South African botanical species had already been commercialised, of which 16 were sourced from indigenous plants, including gum Arabic, baobab, buchu, *waterblommetjies*, rooibos tea, honeybush tea, finger millet, sour fig, jelly melon, palm wine, pearl millet, Livingston potato, marula fruit, sorghum, jugo bean and cowpea (Van Wyk, 2011).

In an article devoted to the potential of South African plants in the development of new food and beverage products, Van Wyk (2011), composed a list more than 120 of indigenous South African species with culinary significance and potential application in food products and suggested some uses in/as beverages, health foods, flavours, herbs and spices, condiments and sweets, for example (Van Wyk, 2011).

Traditionally used seeds, nuts and legumes are favourable because of their ability to grow in dry and poor-quality soil (Van Wyk, 2011). Product development of these raw ingredients could very possibly be aimed at the trending health food and snack market (Van

Wyk, 2011; Rahul *et al.,* 2015). Manketti nuts, marula nuts, marama beans and baobab are some of the better-known examples (Van Wyk, 2011; Rahul *et al.,* 2015).

Researchers have shown that the incorporation of indigenous and traditional food-types into the diet can lead to improved health and welfare of individuals because of the familiarity of the products that supply a range of nutrients (Du Plooy *et al.*, 2018). While improving food security and sustainability, communities are encouraged to consume adequate nutrients as well as their required kilojoules to sustain growth (Du Plooy *et al.*, 2018). Indigenous vegetation supplies easily accessible and culturally acceptable food, promoting nutrition security (Du Plooy *et al.*, 2018).

Indigenous South African fruits serve as a rich supply of vitamins and other nutrients to traditional communities and have the potential to contribute to food security and well-being of South African communities, particularly in dry areas where the cultivation of exotic fruit species is not feasible (Ngemakwe, 2017). The marula fruit is a good example of the inherent potential of these fruit (Van Wyk, 2011). Marula trees are renowned for their prolific bearers of fruit, yielding around 500 kg of fruit per tree per annum, and they are adapted to poor soil quality (Ngemakwe, 2017). In addition, marula fruit boasts a vitamin C content that exceeds that of most citrus fruit (Ngemakwe, 2017).

Despite being the flavour source of Amarula® liqueur, it has also been used to develop delectable sweets (Van Wyk, 2011). In 1996, a study on various health, taste, availability and yield aspects of various southern African fruits recognised the tremendous potential of marula, sourplum, blue sourplum, jacket plum, baobab, *mobola* plum, African mangosteen, forest milkberry, the common cluster fig, green monkey apple and black monkey apple, wild custard apple and wild date palm (Van Wyk, 2011).

While wild vegetables are commonly used as a food source in rural communities, their high nutritional value makes them an ideal food to incorporate into mainstream diets to overcome micronutrient deficiencies and to play a role in food security (Bvenura & Afolayan, 2015). Green leaves of many indigenous plant species are consumed raw or cooked and eaten as an accompaniment to a starch staple (Van Wyk, 2011).

Rooibos tea represents a massively successful form of South African herbal tea, valued because of its delicious, sweet taste, its antioxidant, vitamin, and mineral content, while being caffeine-free (Gruenwald, 2009; Joubert & De Beer, 2011; Van Wyk & Gorelik, 2017). Lesser known, but closely related species from *Aspalathus* have additional unique flavours and have the potential to be developed for niche markets (Joubert & De Beer, 2011; Van Wyk, 2011; Van Wyk & Gorelik, 2017). An example is *A. pendula*, or ‘golden tea’ (Joubert & De Beer, 2011; Van Wyk, 2011; Van Wyk & Gorelik, 2017). It has a golden colour and delightful taste (Van Wyk, 2011).

Honeybush tea is another South African tea that boasts commercial success (Joubert *et al*., 2008; Schulze *et al*., 2015). Its primary phenolic compound, mangiferin, is a xanthone of medicinal significance and has in fact been commercialised as a medicine in some parts of the world (Van Wyk, 2011; Van Wyk & Gorelik, 2017). Many other herbal teas with pleasant aromas and possible health benefits have been identified as teas with potential for commercialisation (Van Wyk, 2011; Joubert *et al.,* 2017). These include bushman's tea (*Athrixia phylicoides*), daisy tea (*Athrixia elata*), 'Hongertee' (*Leysera gnaphoides*), 'ballerja' (*Mentha longifolia*) and 'berg-boegoe' tea *(Myrothamnus flabellifolius*) (Van Wyk, 2011; Joubert *et al*., 2017).

The growth of the herbal tonic industry in SA is largely thanks to *Aloe vera* (Van Wyk, 2011; Maan *et al*., 2017). Polysaccharides are extracted from Aloe leaves using a patented method to produce a sort of gel or 'jelly' that can be used in beverages (Van Wyk, 2011; Maan *et al.*, 2017).

The expanding division of health foods and functional foods opens the door to the development of herbal drinks, botanical extracts and natural flavours, selected on the basis of their health-promoting status as well as their taste and flavour (Van Wyk, 2011). The use of botanical extracts in beverages offers several benefits, whether they are used to create a functional food or purely for their flavour (Gruenwald, 2009).

South Africa is home to an elaborate range of aromatic botanicals, with numerous species offering potential application as novel flavours and fragrances (Van Wyk, 2011). Examples of aromatic plants of particular interest to the food industry include *Heteropyxis natalensis, Mentha longifolia, Myrothamnus flabellifolius, Pelargonium graveolens, Siphonochilus aethiopicus* and *Warburgia salutaris* (Van Wyk, 2011).

Other unexplored fruit-bearing species that could be the source of esters and volatile compounds are *Gethyllis* species, *Osteospermum moniliferum, Parinari curatellifolia, Sclerocarya birrea* to name a few (Van Wyk, 2011).

3. CONSERVATION

With South African plant biodiversity threatened by several factors, it has become essential for effective conservation strategies and implementation (Hills *et al*., 2019). Before local communities can benefit from commercialisation of their traditional resources, such as indigenous plants, research of botanical, horticultural, chemical and nutritional aspects of the sources are required (Welcome & Van Wyk, 2018).

Overharvesting poses a threat to all forms of vegetation and potentially results in a loss of sustainability (Van Wyk & Prinsloo, 2018). No different, medicinal tree species are particularly vulnerable as they are slow-growing, slow to reproduce and often require very

specific growth conditions, therefore limiting their distribution (Van Wyk & Prinsloo, 2018). When these plants die, they are difficult to replace (Van Wyk & Prinsloo, 2018).

In the case of fynbos, primary threats are the expansion of agriculture, urbanisation, uncontrolled fires as well as the growth of alien plant species (Le Maitre *et al*., 1996). Almost a third of the original fynbos area has been lost with 1 200 species critically rare, endangered, or vulnerable (McEwan *et al.,*2014).

Although fynbos offers multiple vital functions and benefits to South Africa, both ecologically and economically, these advantages are not properly understood by many citizens (Le Maitre *et al*., 1996).

Conservation is intertwined with South African socio-economic issues because of the country's history of imperialism and apartheid (McEwan *et al.*, 2014). There has been a policy shift from 'fortress conservation', governed by the landowners, towards community-based conservation (McEwan *et al.*, 2014). For this reason, a National Biodiversity Conservation and Action Plan has been implemented, aimed at the conservation and sustainable utilisation of biodiversity (McEwan *et al.,* 2014).

People affected by the value of biodiversity include both international and local communities, covering a wide range of socio-demographic and age groups (Le Maitre *et al*., 1996). The youth are exceptionally important because through their decisions, they determine the future of their country (Le Maitre *et al*., 1996). As a result of the trend of exponential population growth, pressure on water supplies and the demand for development areas for shelter, agriculture and development is expected to increase significantly in years to come, and without proper education on the importance of plant conservation, biodiversity further will decline (Le Maitre *et al*., 1996).

With the aim of improving the quality of life of its citizens through sustainable development, the South African government implemented the reconstruction and development programme (RDP) in 1994 (Le Maitre *et al*., 1996). Through education focused on local welfare, including information on ecosystems, students will be encouraged to make informed decisions and empowered to participate in a functioning community (Le Maitre *et al*., 1996).

Over-harvesting of vegetation decreases the available resources over time, which will eventually affect the sustainability and by-products (Privett *et al.*, 2014). This will impact the ability to provide to regional and international markets and could eventually lead to species extinction (Privett *et al.*, 2014). Balancing the commercial harvest demand aspects with the conservation of a species, is therefore an essential concept to grasp and calls for information about the ecological and economic effects of harvesting a particular plant (Privett *et al.*, 2014).

To ensure the selection of the most productive genotype for cultivation, botanical studies should be performed (Welcome & Van Wyk, 2018). Similarly, horticultural research

into the best methods of cultivating taxing species or very slow-growing plants, such as fruit trees, may be required (Welcome & Van Wyk, 2018).

Since wild harvesting may not be a viable long-term solution in terms of sustainability, cultivation offers the advantage of a predictable supply of raw materials with enhanced consistency due to the reduction of variation inherent to wild strains (Welcome & Van Wyk, 2018).

Nutritional information on most edible South African plants is sparsely scattered in literature and almost completely absent in international reference sources (Welcome & Van Wyk, 2018). With the goal of attaining commercially viable food products from wild strains, plenty of food science and food engineering research is required to solve unexpected technological post-harvesting problems and to uncover nutritional information (Welcome & Van Wyk, 2018).

To realise Target 9 of the National Conservation Strategy, detailed knowledge on food plants and their wild relatives are essential (Welcome & Van Wyk, 2018). The target calls for the conservation of the genetic variance of crops, their wild relatives and indigenous food plants as well as preservation of indigenous and local customs (Welcome & Van Wyk, 2018).

4. PROCESSING: EXTRACTION TECHNIQUES

As a result of the exponentially increasing demand for plant extracts to use in a variety of food products, supplements, nutraceuticals, and medicines, advanced methods of extraction have been formulated to improve the time, energy and effort efficacy of extractions, as well as the purity and yield of extracts (Mandel & Tandey, 2016; Belwal *et al*., 2018).

Traditional extraction methods include maceration, percolation, digestion as well as preparation of decoctions or infusions (Belwal *et al*., 2018). A more modern technique, developed in the 1700s, known as the “Soxhlet Extraction”, serves as an improved form of digestion and decoction, although it shares many of the disadvantages of conventional extraction methods (Belwal *et al*., 2018). These include the use of large volumes of solvent with low product yield and extensive separation times (Belwal *et al*., 2018).

Novel extraction methods have been developed to keep up with the high product demand with the aim of improving certain aspects of the separation (Belwal *et al*., 2018). These modern methods enhance the efficiency of the separation and selectivity of bioactive compounds (Mandel & Tandey, 2016; Belwal *et al.*, 2018). Novel approaches of extraction involve the application of microwaves, ultrasonic waves, supercritical fluids, enzymes, pressurised liquids, and electric fields, amongst others (Mandel & Tandey, 2016; Belwal *et al.*, 2018). These methods are generally rapid, simple, environmentally friendly, fully automated and render high quality final extracts that are rich in the targeted compounds (Belwal *et al*., 2018).

In order to achieve profitable extractions, optimal design of a systematic approach, combining the botanical or raw product properties (such as its structure, moisture content and particle size) with the technical implementation is necessary (Both *et al.*, 2014; Belwal *et al.,* 2018).

Successful extraction of specific compounds from different botanicals, including polyphenols, alkaloids, anthocyanins, flavonoids, phenolic acids, carbohydrates, polysaccharides, and essential oils requires careful consideration of the physicochemical property of the compound as well as the specific plant part and therefore the nature of the tissue matrix (Belwal *et al*., 2018).

5. SAFETY OF BOTANICALS FOR FOOD USE

5.1 Toxicological safety

Together with the increased interest in the application of botanicals and botanical ingredients in medicines, food products and supplements, there has been greater devotion to researching the safety of these ingredients (Kroes & Walker, 2004). Recorded cases of intoxications have triggered such concerns regarding the safety of botanicals (Kroes & Walker, 2004). Through expanding knowledge, it has become evident that “natural” does not necessarily imply “safe” and that adverse reactions could result from a certain level of intake (Rietjens *et al.,* 2008).

The food industry as well as the national and international health and food safety authorities have recognised the need for safety assessment and policies for novel plants and plant-derived ingredients for their application in foods (Antignac *et al.*, 2011). Consensus was reached that thorough identification and compositional specification are critical to the assessment of the safety of plant-derived ingredients (Antignac *et al.*, 2011). Specific guidance was published for the safety assessment of plant-derived materials used as food ingredients (Antignac *et al.*, 2011). In the same time frame, the European Medicines Agency (EMEA), the US Food and Drug Administration (FDA) and Health Canada published guidelines on the safety assessment of herbal medicines, recognising the necessity of botanical drug identification and characterisation (Antignac *et al.*, 2011).

Botanical food supplements can be sourced from primary food sources, for example soy extracts containing isoflavones or tomato extracts rich in lycopene (Kroes & Walker, 2004). They can also be derived from secondary sources, including herbs and spices, for instance: garlic oil, rosemary extracts or green tea extracts (Kroes & Walker, 2004). Other botanicals have been used as herbal medicines throughout history but have only recently been considered as a food ingredient, such as *Ginkgo biloba*, Ginseng extract and *Hypericum perforatum* (St. John’s Wort) (Kroes & Walker, 2004). Another category of botanicals is those that have no history of previous human use, for example phytostanols derived from wood as a constituent of cholesterol-reducing products (Kroes & Walker, 2004).

Despite the long history of safe use reported for many botanicals, certain botanicals are known to contain toxic, genotoxic or carcinogenic constituents which may become a safety issue at specific levels of exposure (Rietjens *et al*., 2008). Several reported instances of intoxication with botanical products have been linked back to plant misidentification (Schilter *et al*., 2005). Evidently, accurate species identification is vital, especially if the botanical of interest is related to any toxic species (Schilter *et al*., 2005). Similarly, the particular plant organ to be consumed must be defined (Schilter *et al*., 2005). The reason being that the distribution of the toxic compound might not be uniform throughout the plant (Schilter *et al*., 2005).

The hazard assessment of plant extracts is complicated by compositional variability, inherent to natural products (Schilter *et al*., 2005). This is owed to the natural biological variance of plant chemicals as well as the influence of climatic conditions and agricultural practices (Kroes & Walker, 2004). The raw materials are subjected to different extraction methods, resulting in changes to the content of components that affect health and safety of the botanical (Schilter *et al*., 2005).

Additionally, the taste-active or health enhancing component(s) are not necessarily the ones responsible for the risk of adverse effects, and each may be individually affected by environmental influences (Schilter *et al*., 2005). Thus, the safety of the extract is independent of the taste or benefit (Schilter *et al*., 2005). An example of the compositional variation in botanicals can be emphasised using a study of ginseng dietary supplements (Schilter *et al*., 2005). Differences of key constituents among 25 products differed enormously, in fact, up to 200-fold differences were observed (Schilter *et al*., 2005).

As with any substance deliberately added to food, botanicals must be proved to be safe (Kroes & Walker, 2004). With the growing market for botanicals, there is a demand for improved characterisation of the variety of botanicals and botanical preparations for better coordination of the risk assessment of these products (Kroes & Walker, 2004; Rietjens *et al*., 2008). Regulatory actions that have already been implemented to protect consumers against the side effects of botanicals with known safety hazards include implementation of tolerable daily intakes (TDIs), for example with coumarin; applying restrictions on the presence of specific compounds in food and beverage products, as in the case of hydrocyanic acid (HCN), thujone and glycoalkaloids; developing safe upper limits, for example with beta-carotene; communicating to the consumers the risks associated with a specific product, as with St. John's wort and glycyrrhizinic acid; or completely removing hazardous plant varieties and botanical products from the market, these include aristolochic acids, pyrrolizidine alkaloids and kava-kava (Rietjens *et al*., 2008).

Potential legislative frameworks and guidelines to govern the risk assessment of botanical preparations requires accurate characterisation of the botanical as well as any

processing steps it is subjected to, exploring its history of safe use, defining its intended use and estimated level of intake as well as the identification of any hazards in terms of toxicity and genotoxicity (Kroes & Walker, 2004; Rietjens *et al*., 2008). Risk characterisation of botanical products should cover all data available, including predicted human intake on a daily basis, also considering the duration of intake (Kroes & Walker, 2004). Special attention needs to be given to non-target groups and to possible interactions with pharmaceuticals, aspects that are usually not a concern with conventional food additives (Kroes & Walker, 2004).

An important concern is how to manage botanical ingredients that contain potentially harmful chemicals that are either genotoxic or carcinogenic (Rietjens *et al*., 2008). Examples of these compounds include the allyl-alkoxybenzenes estragole, methyl-eugenol, elemicin, tetra-methoxy-alkylbenzene, safrole, myristicin and apiole (Rietjens *et al*., 2008).

Many of these potentially harmful compounds are constituents of many well-known and commonly consumed botanicals and botanical products such as nutmeg, cinnamon, anise star, tarragon, sweet basil, sweet fennel and anise vert as well as products produced from these raw ingredients (Rietjens *et al*., 2008).

The perceived health benefits of botanicals are not taken into account for food supplements since this would make the testing the same as for a medicinal product (Kroes & Walker, 2004). The inherent biological activity of botanical products makes it uncommon that a wide safety margin will be possible (Kroes & Walker, 2004).

To assess the risk coupled to a specific botanical product, a reliable risk assessment method is required (Rietjens *et al*., 2008). The margin of exposure (MOE) assessment method was developed by the European Food Safety Authority (EFSA) to serve as a suitable technique to determine the risk associated with genotoxic and carcinogenic compounds (Rietjens *et al*., 2008). It is currently believed that the intake of such compounds should be minimised to as low as possible and according to the EFSA, carcinogenic and genotoxic substances are not approved as intentional ingredients in food products (Rietjens *et al.,* 2008).

The MOE protocol uses a reference point, defined by experimentation using animals to determine a dosage that induces a cancer response (Rietjens *et al*., 2008). The lower confidence bound of the benchmark dose that results in 10% extra cancer occurrence is used to determine a ratio between this value (the reference point), and the estimated dietary intake (EDI) in humans (Rietjens *et al*., 2008). This MOE approach is a valuable way of setting priorities in risk management of botanical ingredients with genotoxic and carcinogenic properties (Rietjens *et al*., 2008). With this method, it is essential to consider multiple sources of the ingredients and the EDI of each to determine accurate outcomes (Rietjens *et al*., 2008).

An important parameter that cannot be ignored is the effect of the complex food matrix on the bioavailability of the toxic compound of interest (Rietjens *et al*., 2008). Considering the MOE approach, long-term animal exposure to pure substances may not accurately represent

the effect of the compound as a constituent of a food that may affect its absorption (Rietjens *et al*., 2008). A gradual or incomplete release of compounds from a food matrix, the inhibition of intestinal carriers that facilitate the absorption of a compound or bioactivation of analytes through matrix interaction affect the bioavailability the compound of interest significantly compared to the administration of pure doses of the same compound (Rietjens *et al*., 2008). It is therefore necessary to evaluate the possible matrix effects on the bioactivity of the target compounds in botanicals as part of the risk assessment method (Rietjens *et al*., 2008).

Analytical chemistry can be used as the basis of the safety assessment of botanical ingredients with existing databases of toxicological results (Rietjens *et al*., 2008). The method relies on the chemical elucidation of the compounds of interest, grouping according to its structure and evaluation based on existing information on its absorption, metabolism and toxicity (Rietjens *et al*., 2008). Unidentified analytes are evaluated using a conservative threshold of toxicological concern (Rietjens *et al*., 2008).

This technique is founded on the principle that the chemical constituents of botanicals have delimited structural variance because they originate from a limited range of biological pathways (Rietjens *et al*., 2008). Once characterised and assigned to a well-defined congeneric group, the safety evaluation can be determined by the evaluation of the group, therefore reducing the amount of extremely laborious toxicity testing (Rietjens *et al*., 2008).

5.2 Microbiological safety

The microbiological safety of botanicals and botanical preparations is an important safety parameter in these increasingly popular ingredients (Trucksess & Scott, 2008). In many cases, these herbal products are used without knowing the toxicities or safety of compounds in the botanicals (Trucksess & Scott, 2008). Since raw materials for botanical preparations are generally traded in dried form, they are associated mainly with bacterial endospores and fungal spores, capable of surviving low humidity conditions (Warude & Patwardhan, 2004; EHIA, 2008; Fogele *et al*., 2018; Székács *et al.,* 2018). A broad spectrum of viruses as well as bacterial and fungal cells have, however, also been found on plant material (Warude & Patwardhan, 2004). Pathogens are the microbes that deserve the most attention out of these micro-organisms because of their potential detrimental effect on human health (Warude & Patwardhan, 2004; Székács *et al.,* 2018). These microbial contaminants could lead to a toxic product through their ability to convert certain compounds in the plant tissue to harmful substances or through the microbes' ability to produce toxic compounds (Warude & Patwardhan, 2004).

Despite the long history of use of botanicals, information regarding the contamination of these products with moulds and mycotoxins is limited compared to other products on the market (Trucksess & Scott, 2008). Mycotoxins are toxic metabolites produced by certain fungi

on plants in the field or during storage (Warude & Patwardhan, 2004; Trucksess & Scott, 2008). They manifest themselves in different ways; they can be teratogenic, mutagenic and/or carcinogenic and can lead to many different diseases (Warude & Patwardhan, 2004).

Studies on microbial safety on botanicals have indicated the presence of toxigenic moulds, including high levels of *Aspergillus, Penicillium and Fusarium* spp. (Warude & Patwardhan, 2004; Trucksess & Scott, 2008). Although the contamination with mould does not necessarily correlate to contamination with mycotoxins, aflatoxins, ochratoxin A and fumonisins have been detected in botanicals, including medicinal plants and herbal teas (Warude & Patwardhan, 2004; Trucksess & Scott, 2008).

Well known pathogenic microbes commonly associated with botanicals are *Escherichia coli, Listeria monocytogenes, Salmonella typhi, Pseudomonas aeruginosa* and *Staphylococcus aureus* (Warude & Patwardhan, 2004; Chugh *et al*., 2018). Microorganisms associated with herbal products that are most resistant to drying are the spore-forming *Bacillus cereus, Clostridium perfringens, Clostridium botulinum*, and moulds, such as *Aspergillus* spp., and *Penicillium* spp., which are potential producers of a variety of toxins (Witkowska *et al*., 2011; Ainiza *et al*., 2015; Fogele *et al.,* 2018). The ability of *B. cereus* to form endospores enables it to survive various stress conditions, making it a challenging microorganism to eliminate, even during pasteurisation and sanitary practice (Ceuppens *et al.*, 2011; Fogele *et al*., 2018).

Botanical ingredients are often contaminated with toxigenic fungi and pathogenic microbes, originating either from the soil or from the plants themselves, during harvesting or in storage (Trucksess & Scott, 2008). The mycotoxins and endotoxins that these microbes produce pose serious threats to human health and therefore require serious attention (Trucksess & Scott, 2008).

Methods to detect toxins in botanicals include LC (liquid chromatography) with fluorescence or MS (mass spectroscopy) detection, ELISA (enzyme-linked immunosorbent assay) or TLC (thin layer chromatography) (Trucksess & Scott, 2008; Ainiza *et al.,* 2015).

Poor implementation of quality control and lack of monitoring systems could potentially result in the contamination or adulteration of herbal preparations (Chugh *et al*., 2018). Herbs grown in polluted environments (soil, water or air pollution) or farmed negligently, could lead to the production of products that are unsafe for human consumption (Chugh *et al*., 2018). Similarly, inappropriate storage conditions negatively affect the quality of herbal products, especially if they are subjected to fungal or bacterial contamination (Chugh *et al*., 2018). It has been found that under warm, humid conditions, moulds are more likely to produce toxins such as mycotoxins (Trucksess & Scott, 2008). Mould growth can therefore be prevented by implementing strict hygiene and operational guidelines during the harvesting and storage of botanicals (Trucksess & Scott, 2008).

Concerning quality, food supplements are produced according to HACCP (Hazard Analysis and Critical Control Points), whereas herbal medicines are manufactured according to GMP (Good Manufacturing Processes) (Laekeman, 2010). Procedures such as cleaning, drying, and packaging will inhibit or retard microbial growth and toxin production by avoiding conditions that favour their proliferation (EHIA, 2008; Trucksess & Scott, 2008; Warude & Patwardhan, 2004). The European Herbal Infusions Association (EHIA) (2008) provides detailed guidelines as an informative tool to apply Good Agricultural Hygiene Practices (GAHP) and HACCP in the various stages of production of herbal infusions ingredients.

The microbial risks associated with botanical preparations varies depending on the production stage since processing factors contribute to the microbial load of the resulting product (Warude & Patwardhan, 2004; Dao & Dantigny, 2011; Székács *et al.,* 2018). The solvent, temperature, extraction technique and extraction time all significantly affect the microbial load of the botanical preparation (Chugh *et al*., 2018).

6. BAOBAB (*ADANSONIA DIGITATA*)

Adansonia digitata, colloquially known as baobab, is an indigenous southern African botanical, with its raw materials offering a multitude of services in terms of provision of medicine, food, clothing and many more (Gruenwald, 2009; Van Wyk, 2011; Rahul *et al*., 2015).

Recently, Baobab has attracted the interest of many pharmaceutical companies and scientists due to its various traditional uses and has since been classified as a so-called ‘superfruit’, owing to its extraordinary nutritional profile (Kamatou *et al*., 2011; Rahul *et al.*, 2015; Ismail *et al*., 2018). These are fruits that are associated with potential health-enhancing effects thanks to their rich nutritional contents (PhytoTrade Africa, 2009). Considering factors including its abundance, nutritional content, palatability and yield, Baobab was identified as a South African fruit with enormous potential for the development of food and beverage products (Van Wyk, 2011).

The iconic baobab plant is widespread in African countries and is also found in Madagascar, Australia and the Arabian Peninsula (Kamatou *et al.*, 2011; Ismail *et al*., 2018). Six out of eight baobab species are endemic to Madagascar, the likely evolutionary origin of the genus *Adansonia* (Kamatou *et al.,* 2011; Ismail *et al*., 2018).

The African species, *A. digitata* is indigenous to the savannas and savanna woodlands of sub-Saharan Africa (Kamatou *et al.,* 2011). *A. digitata* is commonly found in the southern Africa countries, South Africa, Malawi, Zimbabwe and Mozambique and in the western region of Africa, they are found in Mali, Benin, Senegal, the Ivory Coast, Cameroon and Burkina Faso (Kamatou *et al.,* 2011). Baobabs also grow in the east of Africa, where they are found in countries including Kenya, Uganda and Tanzania (Kamatou *et al.,* 2011).

Baobab trees grow in arid or semi-arid conditions are tolerant to high temperature conditions and can withstand extended periods of drought (Rahul *et al*., 2015). It has, however, been predicted that baobab populations growing in higher rainfall regions, for example certain parts of Malawi, Tanzania and northern Mozambique, are more likely to be stable than those growing in more arid areas, such as in the Limpopo Valley (Venter & Witkowski, 2013).

While baobabs grow in a variety of well-drained soils, ranging from clays to sands, they should have access to sufficient moisture and anchorage (Kamatou *et al.,* 2011). Researchers have showed a correlation between the soil quality in which baobab trees grow and fruit production (Kamatou *et al.*, 2011). It was also discovered that fruit production could be enhanced by a higher clay and crude silt content of the soil (Kamatou *et al.,* 2011).

The baobab tree is characterised by its enormous trunk; in fact, it is classified as the world's largest succulent plant with the diameter of fully-grown trees typically ranging between 10 to 12 m and a height of 23 m or greater (Kamatou *et al.,* 2011; Rahul *et al*., 2015). One of the names used to describe the baobab tree is the "upside-down tree", attributed to the branches' resemblance to the roots of a tree (Figure 2.2) (Kamatou *et al.*, 2011; Rahul *et al*., 2015).

This deciduous tree bears leaves for only three months per year (Kamatou *et al.*, 2011). Essential physiological processes such as photosynthesis occur in the trunk and branches during leafless phases, using water stored in the trunk (Kamatou *et al.*, 2011).

The growth rate of the tree is very slow, likely due to the limited rainfall received (Venter & Witkowski, 2013). It is approximated that it takes between 8 and 23 years before the baobab tree produces seeds, while over 60 years is typical before the tree reaches maturity (Kamatou *et al.*, 2011). At maturity, baobabs are expected to produce between 160 to 250 fruit annually (Kamatou *et al.*, 2011).

The baobab fruit are large and vary in shape, often globose to ovoid or otherwise oblong-shaped (Figure 2.3) (Chadare *et al.* 2009). The irregular fruit are capsule-like with the outer surface covered with velvety, yellowy-green hairs (Chadare *et al*., 2009; Kamatou *et al.*, 2011; Ismail *et al.*, 2018). The "dead-rat tree" is another informal name belonging to the baobab, describing the appearance of its indehiscent fruit (Chadare *et al*., 2009; Rahul *et al*., 2015). The endocarp, or the inside of the fruit, is segmented into small, powdery, dehydrated slices containing the seeds and filaments (Figure 2.3) (Phytotrade Africa, 2009). The fruits grow up to 12 cm in length and enclose large seeds embedded in the white, acidic fruit pulp (Rahul *et al*., 2015; Ismail *et al*., 2018). The smooth seeds are reniform in shape and dark brown to reddish-black in colour (Figure 2.3) (Chadare *et al*., 2009; Rahul *et al.,* 2015). Beautiful, large white flowers hang from the baobab tree from October to December (Kamatou *et al.*, 2011).

Living up to its nickname as the “chemist tree”, almost every part of the baobab plant has an established purpose in traditional medicine in Africa, varying amongst the different countries (Kamatou *et al.*, 2011; Rahul *et al*., 2015). Baobab has been thought of as a panacea, or a ‘cure-all’ by traditional healers, with documented use against a host of ailments, including treatment of malaria, tuberculosis, fever, microbial infections, diarrhoea, anaemia, dysentery and toothache, amongst others (Kamatou *et al.,* 2011; Rahul *et al*., 2015).

While typically known for their edible fruit pulp, the leaves, seeds, flowers, bark and roots are all edible and have been traditionally consumed in one form or another (Rahul *et al.,* 2015). The leaves, fruit pulp and seeds can be used in sauces, porridges and beverages (Kamatou *et al.*, 2011). The fruit pulp can also be used as seasoning or as an appetiser, in fruit juices, snacks, sweets, or as a fermenting agent in local brews (Kamatou *et al.*, 2011; Muthai et al., 2017).

Considering the increasing demand for protein and energy to support the ever-growing world population in mind, researchers have directed their efforts at exploring new, unconventional food sources, especially those that thrive in arid and semi-arid conditions (Osman, 2004). Sparked by the array of traditional uses, researchers have investigated the nutritional properties of baobab as a food ingredient (Rahul *et al.,* 2015). This led to the uncovering of the exceptional nutritional profile of the baobab products (Osman 2004).

The fruit pulp supplies on average (in g.100 g^{-1} dry weight) an abundance of carbohydrates (74.9), soluble and insoluble dietary fibers (13.7), a low crude protein content (5.3) and a very low fat content (3.6) (Osman, 2004; Chadare *et al.*, 2009; Rahul *et al*., 2015). The noticeable sweet taste of the baobab fruit pulp is owed to the presence of sugar (Chadare *et al*., 2009). According to a study by Murray *et al*. (2001), simple sugars in baobab pulp account for approximately 36% of the total carbohydrates (Chadare *et al*., 2009). It has a slightly acidic taste, which is owed to the organic acids present, including citric, tartaric, malic and succinic acid (PhytoTrade Africa, 2009).

The fruit pulp is rich in potassium, calcium, magnesium, and phosphorus and is a great source of iron, zinc, and copper (Osman, 2004; Chadare *et al*., 2009; Rahul *et al*., 2015). The average mineral content of the fruit pulp (mg.g^{-1} dry weight) is: Potassium (1 794), calcium (302), phosphorus (96-118) and magnesium (195). The fruit pulp boasts an average iron content of 4.3 mg.100 g^{-1} dry weight (Chadare *et al*., 2009).

The baobab fruit pulp is an ideal beverage ingredient, with its pleasant, tart taste, water solubility and exceptional antioxidant and anti-inflammatory properties, as well as its ability to act as a prebiotic ingredient (Gruenwald, 2009). Possible application ranges from soft drinks, natural fruit smoothies and juices to fermented milk drinks, fruit fillings, jams, sauces and desserts (Gruenwald, 2009; Rahul *et al.*, 2015).

Arguably the most sought-after trait, characteristic of the baobab fruit pulp, is its phenomenally high content of ascorbic acid and dietary fibre (Kamatou *et al.*, 2011). The levels of vitamin C contained in the baobab fruit varies significantly, with documented values ranging from 150 to 500 $mg.100^{-1}$ g^{-1} of dry weight (Chadare *et al*., 2009). It is noteworthy to compare this value to the average vitamin C content of oranges (51 $mg.100\ g^{-1}$) (Kamatou *et al.*, 2011; Rahul *et al*., 2015).

Baobab dried fruit pulp is high in dietary fiber, with reported instances reaching percentages greater than 44% of the fruit (PhytoTrade Africa, 2009; Garvey *et al.,* 2017). The high level of the indigestible soluble fiber, pectin, ranges from 23 to 30 $g.100\ g^{-1}$ in the pulp (PhytoTrade Africa, 2009). Pectin is useful in the food industry as a gelling or thickening agent and stabiliser in food (PhytoTrade Africa, 2009). Soluble fiber has also gained interest due its contribution to enhanced satiety as well as its role in promoting gut-health and the reduction of total and LDL (low density lipoprotein) cholesterol (PhytoTrade Africa, 2009; Garvey *et al.,* 2017).

Phenolic compounds are phytochemicals that have been identified as essential bioactive compounds contained within the pulp because of their antioxidant, anti-inflammatory, and antimicrobial properties (Chadare *et al*., 2009; PhytoTrade Africa, 2009; Ismail *et al*., 2019). Various phenolic compounds have been linked to the baobab fruit, including procyanidin, epicatechin, gallic acid, hydroxycinnamic acid glycosides as well as the major contributors to the antioxidant capacity: tannins, phenols and flavonoids (Ismail *et al*., 2019).

The most commonly occurring of the 46 liquid chromatography–mass spectrometry–quadrupole time-of-flight (LC-MS-QTOF) identified phytochemicals in the pulp were proanthocyanidins, phenolic acids, flavonols and saponins (Ismail *et al*., 2019).

The same researchers investigated the best extraction technique for baobab fruit pulp based on the extraction capacity and selectivity of solvents with varying polarities for phenolic and flavonoid contents as well as antioxidant activity (Ismail *et al*., 2019). The solvent, 80% acetone was decided as the preferred solvent choice for the extraction of phytochemicals from baobab fruit pulp (Ismail *et al*., 2019). The method of extraction was as follows: 1 g of baobab fruit pulp was combined with 10.00 mL of solvent before being sonicated for 30 minutes at 30°C (device frequency at 40 kHz and ultrasonic input power at 250 W) (Ismail *et al.*, 2019). The samples were further centrifuged at 5 000 g, 20°C for 15 min and filtered with a 0.22 µm syringe filter for high performance liquid chromatography (HPLC) analysis (Ismail *et al.*, 2019).

A noteworthy finding by Ismail *et al.* (2019) was that water, used as a component, or as a whole, proved to be a good solvent for the extraction of phenols, suggesting the presence of significant proportion of polar phenolic compounds in baobab fruit pulp. The addition of water to organic solvents significantly increases the amount of the total phenolic content

(TPC), particularly when combined with methanol, ethanol, and acetone, as confirmed by other studies (Ismail *et al.*, 2019).

In an evaluation of the integral antioxidant capacity of extracts of *Adansonia digitata*, photochemiluminescence (PCL) was used to determine the integral antioxidant capacity (IAC), that is the combined water and lipid-soluble antioxidant strength (Besco *et al*., 2007; PhytoTrade Africa, 2009). In the water-soluble component, antioxidants such as flavonoids and ascorbic acid were detected, whereas in the lipid-soluble component, tocopherols and carotenoids were detected (PhytoTrade Africa, 2009).

The water-soluble antioxidant capacity of baobab fruit pulp was 6.96 mmol equivalents of Trolox.g^{-1} whereas the lipid-soluble fraction was slightly lower, at 4.148 mmol equivalents of Trolox.g^{-1} (PhytoTrade Africa, 2009).

Besco *et al*. (2007) showed that the baobab fruit pulp exibited the greatest overall antioxidant activity compared to other vitamin C-rich fruits (Besco *et al*., 2007). Baobab fruit pulp IAC was followed by the IAC of the fresh pulp of oranges, strawberry, bilberry and lastly kiwi fruit (Besco *et al*., 2007). The water- and lipid-soluble antioxidant capacity, or IAC, of the baobab fruit pulp was reported as 240.5 µmol.g^{-1} (Besco *et al*., 2007).

As with most fruits, baobab fruit pulp naturally contains antinutrients that can hinder the availability of the useful nutrients (Osman, 2004; Chadare *et al*., 2009). These include trypsin inhibitors, phytates and tannins (Chadare *et al.,* 2009). Trypsin inhibitor activity (TIA) analyses showed a low level in the pulp (5.8 TIA.mg^{-1} sample) (Osman, 2004). Phytic acid (2.6 mg.100 g^{-1}) as well as tannin concentrations were also low in fruit pulp (Osman, 2004). The tannin content of the pulp was approximated as 0.0051% and 0.0062%, a level too low to be of detriment to consumers (Chadare *et al.,* 2009).

Other potentially hazardous compounds detected in baobab pulp include HCN (0.0049% dry weight) and total oxalate (0.0044% dry weight), however, these levels are far below the concerning safety levels (Chadare *et al.,* 2009).

In 2008, the European Commission authorised the import of dried baobab fruit pulp as a Novel Food (Hermann, 2009; Kamatou *et al.*, 2011). One year later, the FDA accepted baobab fruit pulp as a food ingredient in the United States of America (Kamatou *et al.*, 2011). The FDA's decision was that baobab (*A. digitata*) dried fruit pulp is generally recognised as safe (GRAS) and accepted its use as a food ingredient in blended fruit drinks and fruit cereal bars at levels of up to 10% and 15%, respectively (Kamatou *et al.*, 2011).

Although PhytoTrade reasoned that the family *Malvaceae*, under which baobab is classified, as well as the related species of the family *Bombaceae,* are generally free from toxic or allergenic substances, EFSA panel strongly suggests the routine quality control analysis for aflatoxins since the fruit could be subjected to storage conditions that favour mould growth (Hermann, 2009).

The low-moisture content (10-12%) of naturally dehydrated ripe fruit, as well as the acidic nature of the pulp, with a pH value of around 3.3, are both common microbial hurdles (Chadare *et al*., 2009; UNCTAD, 2005). In addition, baobab plant parts have been commonly used in history to treat microbial infections (Kamatou *et al.*, 2011). The antibacterial activity, attributed to the presence of tannins, phlobatannins, terpenoids and saponins also potentially inhibit microbial growth in the pulp (Kamatou *et al.*, 2011; Ismail *et al*., 2019). Considering these parameters, the fruit pulp of baobab is presented as a low-risk product concerning microbial hazards.

Since baobab products are becoming increasingly commercialised and exported, pressure on this resource is also increasing (Kamatou *et al.*, 2011). Due to the high demands, the baobab tree needs to be conserved (Kamatou *et al.*, 2011). To prevent over-exploitation of the plant, successful cultivation of this commercially important tree is of the essence (Kamatou *et al.,* 2011).

The outcomes of the ecological modelling of baobab growth indicated that cultivation is theoretically possible in most southern African countries as well as in West Africa (Kamatou *et al.,* 2011). Because of its reputation for growing slowly, the baobab tree has not been commonly domesticated (UNCTAD, 2005). Cultivation requires that the seeds be treated to break their dormancy before sowing, as if passing through animal's gut (UNCTAD, 2005). Soaking the seeds in sulfuric acid for 6 to 12 h, followed by rinsing in water for another 24 h resulted in germination success of greater than 90% (UNCTAD, 2005). Direct sowing has a very low success rate of around 10% (Kamatou *et al.*, 2011).

Grafting can be very useful in baobab cultivation, enabling the selection of desirable characteristics from adult trees and developing new trees faster than trees grown from the seed (Kamatou *et al.*, 2011). Other advantages of grafted plants are smaller trees for easier fruit harvesting as well as shortened time until flowering (UNCTAD, 2005). One draw-back is a lower fruit yield (30%) (UNCTAD, 2005)

The existence of variation in traits of baobab trees across the several countries that support their growth serves as an opportunity for the selection of optimal adult trees for domestication, breeding, and conservation (UNCTAD, 2005; Muthai *et al*., 2017). Indigenous knowledge of parameters including nutritional composition, taste and growth patterns could guide farmers in their selection of appropriate tree candidates for propagation (Kamatou *et al.*, 2011).

Figure 2.2 Mature *Adansonia digitata* tree (Siyabona Africa, 2017)

Figure 2.3 Fruit and seeds of *Adansonia digitata* (Rahul *et al.,* 2015)

7. MORINGA (*MORINGA OLEIFERA*)

Moringa oleifera is another one of the numerous African medicinal plants that has been recognised due to its enormous potential as a phytopharmaceutical as well as a dietary supplement (Liu *et al.*, 2018; Matic *et al*., 2018). This nutrient-rich superfood has also been identified as a sustainable crop to help combat food insecurity (Matic *et al*., 2018; Sivakumar *et al.*, 2018; Welcome & Van Wyk, 2018).

M. oleifera is one of the most-studied and utilised species, owing to its phytochemical profile and health properties (Ma *et al*., 2018). The full potential of *M. oleifera* is yet to be fully explored in the food and beverage sector (Oyeyinka & Oyeyinka, 2015).

Although native to India, Pakistan, Bangladesh and Afghanistan, the Moringa tree is adapted to arid conditions, and thus grows well in western and eastern Africa, South Africa, Tropical Asia and Latin America (Karim *et al*., 2016; Matic *et al*., 2018). In South Africa, *M. oleifera* was introduced for cultivation in 2006 and has since slowly gained popularity, especially in the savanna and grasslands of Gauteng, Limpopo, Mpumalanga and KwaZulu-Natal provinces (Tshabalala *et al*., 2019).

M. oleifera has been used in herbal medicine in India and Africa for centuries (Karim *et al*., 2016; Matic *et al*., 2018). Used traditionally as a panacea, it is thought to cure hundreds of ailments, acting as an antioxidant-, anticancer-, anti-inflammatory-, antidiabetic- and an antimicrobial-agent (Leone, *et al*. 2015, Liu *et al*., 2018; Matic *et al*., 2018). While many cultures and communities believed in the healing properties of Moringa based on first-hand experiences, their trust is gradually being confirmed by recent scientific and clinical evidence (Vergara-Jimenez *et al.*, 2017; Matic *et al*., 2018). Some of the claimed pharmacological properties, supported by research, includes its anti-bacterial, antidiabetic, anti-dyslipidemic and anti-tumour properties (Stohs & Hartman, 2015; Karim *et al*., 2016). It is thus not surprising that the tree is often referred to as the "Nebedaye", meaning "never die" tree, or "the miracle tree" in many African languages (Matic *et al*., 2018).

Moringa is the only genus belonging to the *Moringaceae* family (Leone *et al*., 2015; Vergara-Jimenez *et al.*, 2017). The genus is divided into 13 species, each with unique characteristics and conditions that favour their growth (Leone *et al*., 2015). The most widely cultivated species is *Moringa oleifera* (Leone *et al*., 2015; Vergara-Jimenez *et al.*, 2017).

Moringa oleifera is a rapid-growing, soft-wood tree, reaching heights of 5-12 m (Leone *et al*., 2015; Vergara-Jimenez *et al.*, 2017). The perennial, deciduous, cruciferous tree grows in tropical and subtropical conditions with dry to moist climate, with annual rainfall anywhere from 760 to 2 500 mm and temperatures of around 18°C to 28°C (Leone *et al*., 2015; Ma *et al*., 2018). These trees survive any soil type, except for heavy clay and waterlogged soils and prefer soil pH between 4.5 and 8.0, at altitudes of up to 2 000 m (Leone *et al.,* 2015).

The tree, *M. oleifera* can be identified by tripinnate leaves, with either white or yellow petioles as well as hanging 3-sided pods, the characteristic behind the nickname, "the drumstick tree" (Figure 2.4) (Stohs & Hartman, 2015; Gupta *et al.*, 2018; Matic *et al*. 2018). Other properties include its whitish-grey, corky bark, globular, winged seeds, its tuberous taproots as well as its bisexual, stalked, axillary flowers that are white or cream in colour (Gupta *et al.,* 2018).

All of its plant parts are edible and nutritious, including the flowers, pods, roots, leaves and seeds (Stohs & Hartman, 2015; Liu *et al.*, 2018; Ma *et al.,* 2018). The most commonly used parts of the plant are, however, the leaves, which are rich in bioactive compounds and exhibit the greatest antioxidant activity (Vergara-Jimenez *et al.*, 2017; Matic *et al*., 2018). The

high antioxidant activity holds a direct relationship with the anticancer potential of *M. oleifera* leaves due to the protection of major biomolecules from free radical-related oxidative damage (Liu *et al*., 2018).

Countless nutritional studies have been performed on the parts of the *M. oleifera* tree, agreeing that the leaves are an excellent source of proteins, minerals and vitamins (Karim *et al*., 2016; Vergara-Jimenez *et al.*, 2017; Matic *et al*., 2018). Reported protein contents for dried *M. oleifera* leaves range from 19 to 35 g.100 g^{-1} dry weight in research (Stohs & Hartman, 2015; Olsen *et al*., 2016; Saucedo-Pompa *et al*., 2018).

Compared to other well-known nutrition references, on average, 100 g of dried *M. oleifera* leaves contained 12 times more vitamin C than oranges, 10 times the vitamin A content of carrots, 9 times more protein than yoghurt, 15 times the potassium content of bananas, 17 times the calcium content of milk and 25 times more iron than spinach (Karim *et al.,* 2016; Matic *et al*., 2018).

The health-promoting properties of the leaves can be attributed to numerous bioactive compounds present, including tannins, saponins, steroids, glycosides, quercetin, terpenoids, gallic acid, caffeic acid, phytosterols amongst many others (Matic *et al.,* 2018). Chlorogenic acid, quercetin and kaempferol are prominent in *M. oleifera* leaf extracts (Liu *et al.*, 2018). These abundant secondary metabolites in the leaf extracts have been linked to the reduction of chronic diseases and offer multiple benefits including activity as an antioxidant-, anti-tuberculosis-, analgesic-, anti-cancer-, anti-diabetic-, antispasmodic-, diuretic-, antihypertensive-, cholesterol lowering-, antimicrobial- and antimalarial-agent (Liu *et al*., 2018; Matic *et al*., 2018).

Other minerals, including zinc, magnesium, and copper, and vitamins such as vitamin B, folic acid, pyridoxine and nicotinic acid, vitamin D and E are also abundant in *M. oleifera* (Matic *et al*., 2018).

Fresh and dried *Moringa* leaves have formed an integral part of the cuisine in African countries for many centuries (Oyeyinka & Oyeyinka, 2016). The leaves are consumed as a vegetable, in a similar way to spinach or to prepare soups, sauces and stews (Matic *et al*., 2018). Dried leaves are often milled and added as a seasoning agent (Matic *et al*., 2018).

A more recent application of *Moringa* that has received much interest is its use as a food fortificant (Oyeyinka & Oyeyinka, 2016). Applications such as weaning foods, ‘amala’, biscuits, bread, cake, cheese, soups, cereals and yoghurt have been explored (Oyeyinka & Oyeyinka, 2016). The outcome was that the nutritional properties were improved at the cost of the sensory properties (Oyeyinka & Oyeyinka, 2016). The colour, taste and aroma were negatively impacted by the addition of high concentrations of leaf powder, likely due to its bitter, herbal taste and green colour (Oyeyinka & Oyeyinka, 2016).

Extraction techniques to maximise the flavonoids and phenolic compounds from dried Moringa leaves have been explored using water, ethanol, and methanol (Lin *et al.,* 2018). It was found that the total flavonoid content of ethanol and methanol exceeded the pure water extractions (Lin *et al.*, 2018). Additionally, the total phenolic and total flavonoid concentrations were greater in 70% ethanol compared to 50% ethanol (Lin *et al.*, 2018). While both methanol and ethanol extraction yield greater flavonoid and phenolic concentrations, ethanol is generally preferred since methanol poses a threat to environmental as well as human health (Lin *et al.,* 2018).

In a different study, subcritical ethanol extraction of flavonoids from *Moringa oleifera* leaves proved to be an efficient method for large scale extractions (Belwal *et al*., 2018). The conditions were optimized at a temperature of 126.6°C, using an extraction time of 2.05 h and 70% ethanol (Belwal *et al*., 2018). The subcritical extraction required only 4 h to obtain the same flavonoid concentration as that obtained by at least half a day of the conventional ethanol reflux, thereby saving a large amount of energy (Belwal *et al*., 2018).

As a result of growing popularity of *M. oleifera* applications in the food industry, safety in terms of toxicity have recently been explored (Stohs & Hartman 2015; Lin *et al.*, 2018). Various *in vitro* safety studies concerning aqueous leaf extracts indicate a high degree of safety (Stohs & Hartman, 2015). Dose-response evaluation of the aqueous leaf extract in rats to concluded that an intake of up to 2 000 $mg.kg^{-1}$ body weight is considered safe while the recommended daily consumption should not exceed 70 g (Tiloke *et al.*, 2018).

Investigation of sorption isotherm behaviour of *M. oleifera* leaf powder showed an increase in moisture content with the increase of water activity and the curve was characterised by a sigmoidal form, similar to the behaviour of other medicinal and aromatic plants (Rébufa, Pany & Bombarda, 2018). For water activity values below 0.5, the content of available free water was almost constant (8–10%), which shows that *M. oleifera* dried leaf powder is microbiologically stable and consequently has extended shelf-life (Rébufa, Pany & Bombarda, 2018).

Increasing consumer health awareness has driven the market for natural, nutrient-rich foods, resulting in the growing demand for superfoods such as *M. oleifera* (Karim *et al.*, 2016).

As a result of increased pressure on natural resources, conservation of the species for ethnobotanical, pharmacological, nutraceutical and biodiversity purposes is essential (Gupta *et al.,* 2018).

Fortunately, Moringa is easily cultivatable via sowing or cutting and is resistant to conditions of drought, poor soil quality as well as mild frost (Leone *et al*., 2015; Gopalakrishnan *et al.*, 2016). Sowing offers the advantage of limited labour requirement, with a high germination rate of 80%–90%, provided ideal storage conditions are adhered to, namely 3°C, 5%–8% moisture (Leone *et al*., 2015). Cutting is more labour-intensive but is preferred when

seed availability is scarce (Leone *et al*., 2015). Research, however, suggests that trees grown from seeds develop longer roots than those originating from cuttings, providing enhanced stabilisation and better access to water (Leone *et al*., 2015).

Due to the fast-growing nature of the tree, *M. oleifera* grows to approximately 3 m in height within three months and 12 m high in a couple of years (Leone *et al*., 2015). Pruning or pollarding enhances lateral branching to maximise the leaf harvest (Leone *et al*., 2015).

Leone *et al*. (2015) discusses the potential of the natural variation within the *Moringa* genus as a source for its improvement, emphasising the need for germplasm banks to conserve the genetic variability present in Moringa (Leone *et al*., 2015).

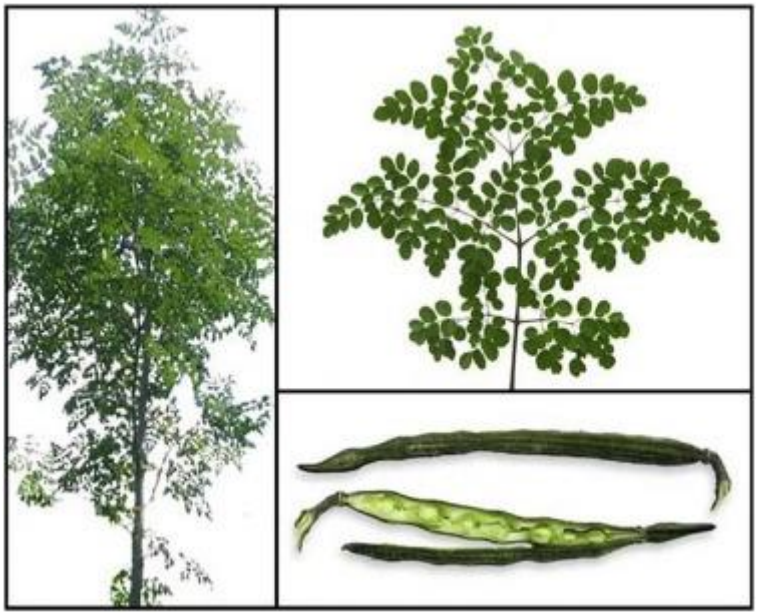

Figure 2.4 *Moringa oleifera* tree, leaves and pods (Tiloke *et al*., 2018)

8. HONEYBUSH (*CYCLOPIA GENISTOIDES*)

Honeybush is a traditional South African tea, derived from the leaves, stems and flowers of a variety of *Cyclopia* species (De Nysschen *et al.,* 1996; Theron *et al*., 2014). The Cape-endemic *Cyclopia* genus belonging to the *Fabaceae* family is comprised of 23 species of papilionoid legumes (Stepanova *et al*., 2012). The name, 'honeybush' emanates from the sweet, honey-like scent of the shrub in full bloom (Joubert *et al.,* 2011).

Cyclopia spp. are found on the southern slopes of mountainous and coastal parts of the Eastern and Western Cape regions of South Africa (Joubert *et al*., 2011; Brink *et al*., 2017). The growth of certain honeybush species are widespread while others are limited to smaller areas (Brink *et al*., 2017). Wetter and cooler conditions are preferred by species that grow on the southern slopes of the mountainous regions (Brink *et al*., 2017). These plants have become fire-adapted via either sprouting or seeding (Brink *et al*., 2017). *Cyclopia* spp. thrive

in the low pH, well-drained, sandy-loam, infertile soils associated with the fynbos biome through their specialised root systems (Brink *et al*., 2017).

Cylopia spp. rely on rhizobia in their root nodules for nitrogen fixation as well as a supply of essential nutrients such as phosphorous, in exchange for a carbon source (Brink *et al*., 2017). *Cyclopia* spp. are long-lived perennials and reach heights of up to 3 m in favourable conditions (Joubert *et al*., 2011).

A variety of *Cyclopia* species are used for herbal tea brewing, with the retail product generally consisting of a blend of species (Theron *et al*., 2014; Schulze *et al.,* 2015). Honeybush tea is currently prepared mainly from the three species, namely, *Cyclopia genistiodes, C. intermedia* and *C. subternata,* all of which are grown commercially (De Nysschen *et al*., 1996; Theron *et al.,* 2017). Provided that all *Cyclopia* species can be used to produce honeybush tea, it has been proposed that alternative species with unique flavours can be used to extend the range of honeybush sensory profiles as the demand continues to grow (Theron *et al.,* 2014; Van Wyk & Gorelik, 2017).

The morphological and chemical properties of the species hold many similarities (Stepanova *et al*., 2012). The plants are either sprouting or non-sprouting woody shrubs with yellowy-twigs, trifolate leaves, small, yellow flowers and brown pods containing arillated seeds (Stepanova *et al*., 2012).

South African herbal teas have infiltrated the global market during recent decades, in tandem with the growing international herbal tea market, driven by consumer awareness of their health properties (Schulze *et al*., 2015; Joubert *et al.,* 2017). Scientific evidence of the perceived health benefits associated with herbal teas has also become more readily available (Joubert *et al*., 2017).

Honeybush represents a concentrated source of phenolic compounds that are scarce in other plant-based food and beverage products (Schulze *et al*., 2015). The raw material of *Cyclopia* species is usually commercially oxidised before it can be enjoyed in its traditional fermented form (Stepanova *et al.,* 2012; Schulze *et al*., 2015). Fermented honeybush tea is rich in polyphenols belonging to the xanthone, benzophenone, dihydrochalcone, flavanone and flavone phenolic subclasses (Schulze *et al.,* 2015; Joubert *et al*., 2017). The xanthone, mangiferin, and the flavanone glycoside, hesperidin, are two of the primary *Cyclopia* phenolic compounds, both with well-established health-benefits (Schulze *et al.,* 2015; Stepanova *et al*., 2012; Van Wyk & Gorelik, 2017). Mangiferin exhibits a broad range of medicinal properties, including antioxidant-, anti-inflammatory and anti-diabetic properties, among others (van der Merwe *et al*., 2017). Hesperidin has been identified as an effective anti-inflammatory, anti-carcinogenic and antioxidant compound, as confirmed by several studies (Devi *et al.,* 2015).

Another major contribution to honeybush's popularity, as with rooibos tea, is owed to its caffeine-free nature as well as its low tannin content (Brink *et al*., 2017; Stander, Joubert &

De Beer, 2019). It also contains appreciable amounts of the sugar alcohol, pinitol, which acts as an expectorant and possesses possible anti-diabetic effects (Joubert *et al.*, 2007).

The use of *Cyclopia* spp. to prepare herbal tea has well-documented use in the Cape, dating back to the 19th and early 20th centuries (Van Wyk & Gorelik, 2017). The first reference to honeybush was found in a 1705 European taxonomic script (Joubert *et al*., 2011). Many other records have been found, including a description by Bowie in 1830, describing the tea as an astringent decoction, often used by the Colonists as a restorative (Van Wyk & Gorelik, 2017).

As with rooibos, the use of honeybush was initiated by its medicinal properties and subsequently transitioned to non-medicinal use, in other words, herbal tea enjoyed for its taste (Joubert *et al*., 2011). However, the modern approach of "food as medicine" has brought emphasis back to the medicinal properties (Joubert *et al.,* 2011).

Although the honeybush industry is thought of as a developing industry considering the slow rate of honeybush commercialisation in the past 100 years, it is expected that honeybush will become an increasingly significant South African export product (Joubert *et al*., 2011; Van Wyk & Gorelik, 2017). While sensory appeal remains the primary consumer driver of choice for most herbal teas, the pharmacological properties of beverages have become a determining factor in purchasing decision of health-orientated consumers (Joubert *et al.*, 2017). Therefore, market opportunities are growing for herbal teas (Joubert *et al.*, 2017).

Traditionally, the leafy shoots and flowers are fermented and sun-dried to prepare tea (Joubert *et al*., 2008; Joubert *et al*., 2011). Today, the primary commercial product is still in the fermented form (Stepanova *et al*., 2012). Since the traditional heap fermentation method enabled very little control over production parameters, in addition to the high demand for high quality tea and strict export regulations regarding microbial contamination, alternative processing techniques had to be developed (Joubert *et al*., 2008).

Commercially, the plant material is mechanically cut into small pieces and moistened to approximately 65% moisture content before being fermented (80°C for 24 h or 90°C for 16 h) and dried under controlled conditions (40°C for 6 h) (Joubert *et al.*, 2008; Theron *et al.,* 2014). Its distinctive dark brown colour and characteristic sensory profile are obtained during a high-temperature oxidation process (Erasmus *et al*., 2017). It has been shown that high temperature fermentation and drying under controlled conditions improves the sensory quality of the tea and that temperatures ≥ 60°C adequately inhibits the growth of thermophilic moulds (Joubert *et al*., 2008). High temperature fermentation requires the addition of water before fermentation, thereby improving the uniformity of the colour of the final product and enhances the release of the tea solids when preparing the infusion (Joubert *et al.*, 2008).

Large variation in the sensory quality of samples, sourced from different commercial processors, indicated to a large extent the effect of processing conditions on the aroma and

flavour achieved during fermentation (Stepanova *et al*., 2012; Erasmus *et al*., 2017; Bergh *et al*., 2017). Erasmus *et al.* (2017) investigated the influence of the oxidation temperature and time on the sensory characteristics of *Cyclopia genistoides, C. subternata, C. maculata and C. longifolia*. The aim of the study was to minimise negative sensory attributes while enhancing the desirable sensory traits (Erasmus *et al*., 2017). Fermentation parameters of 80°C and 90°C for 8, 16, 24 and 32 h were applied after which descriptive sensory analysis was used to detect changes in aroma, taste and mouthfeel (Erasmus *et al.,* 2017). The major defining aroma attributes of honeybush remained consistently high throughout the oxidation trials while positive aroma attributes contributing to unique species profiles changed with fermentation conditions (Erasmus *et al.,* 2017). Good and bad sensory attributes were intensified and reduced, without introducing new aromas (Erasmus *et al.,* 2017). The predominant trend with increasing fermentation time was that the "sweet" and "floral" notes increased while the "green" notes decreased, especially at times exceeding 16 h (Erasmus *et al.,* 2017). Considering the sensory effects as well as industrial costs, fermentation time should not exceed 24 h while small manipulations of the positive aroma profiles can be achieved by adjusting the fermentation conditions from 80°C/24 h to 90°C/16 h (Erasmus *et al*., 2017).

In an investigation of the phytochemicals present in *C. genistoides*, an aqueous extraction technique that proved to be suitable was to mill the dried plant material using a Retsch mill (1 mm sieve; Retsch GmbH, Haan, Germany) and exhaustively extract the botanical at 93°C for 30 min, using a 1:100 ($w.v^{-1}$) plant powder to water ratio (Schulze *et al*., 2015). Aliquots were preserved at −20°C until subsequent use for HPLC-DAD (Schulze *et al*., 2015). Before LC analyses, aqueous ascorbic acid was added to the defrosted extracts to yield a final concentration of *ca.* 9 $mg.mL^{-1}$, protecting the sample from oxidation (Schulze *et al*., 2015). The samples were filtered before analysis, using 33 mm diameter, 0.22-µm pore size filters (Schulze *et al*., 2015).

It is natural for polyphenols to decrease under elevated temperature conditions, as experienced by honeybush during fermentation. In an attempt to conserve the health-promoting constituents, unfermented, or green honeybush tea was studied, leading to a patented process for vacuum drying to produce green honeybush tea (Joubert *et al*., 2011).

Typical sensory descriptors of fermented honeybush, established for infusions of several *Cyclopia* species (*Cyclopia sessiliflora, C. longifolia, C. genistoides, C. intermedia, C. subternata* and *C. maculata*), includes the terms "floral", "fruity", "plant-like", "woody" and "sweet-associated", with a sweet taste and slight astringent mouthfeel (Stepanova *et al*., 2012; Erasmus *et al.,* 2017). Other properties contribute to differences in the sensory characteristics inherent to specific *Cyclopia* species (Stepanova *et al*., 2012; Erasmus *et al.,* 2017). A generic sensory wheel was established by Theron *et al.* (2014), in which 30 attributes are organised into positive and negative traits in primary and secondary tiers (Figure 2.5). The species could

be categorised into three distinct groups, namely group A, comprised of *C. sessiliflora , C. intermedia* and *C. genistoides*, characterised by their “fynbos-floral”,“ fynbos-sweet” and “plant-like” attributes, group B, consisting of *C. longifolia* and *C. subternata* due to their descriptors, “rose geranium” and “fruity-sweet” and group C, containing *C. maculata*, with “woody”, “boiled syrup” and “cassia/cinnamon” as sensory descriptors (Theron *et al.,* 2014). The benefit of unique sensory profiles associated with various species is that they could potentially be used to establish niche markets (Stepanova *et al*., 2012). The honeybush sensory wheel enables flavour description of honeybush tea as an important quality control tool to improve product consistency and thus consumer confidence (Figure 2.5) (Stepanova *et al*., 2012; Van Wyk & Gorelik, 2017).

Concerning safety aspects of honeybush consumption, no reports of toxicity have been recorded (Joubert *et al*., 2008). Chronic consumption of aqueous extracts of unfermented and fermented honeybush by rats over a period of 10 weeks did not cause any harm to their well-being (Joubert *et al*., 2008). While subsequent studies are in place, consumption is assumed to be safe considering its long history of use (Joubert *et al*., 2008).

Regarding microbiological safety, both fermented and green (unfermented) export tea must conform to the regulatory standards of the Department of Agriculture as administered by the Perishable Products Export Control Board (Joubert *et al*., 2011). Parameters including cut size, moisture content, foreign matter contamination, pesticides as well as microbial quality are described by the Agricultural Product Standards Act 119 of 1990 (Government Notice No. R 1177 of 24 Nov 2000 and amended according to Notice No. R 1132 of 15 July 2005) (Joubert *et al*., 2011).

The relevant safety standard requires the plant material to be free from *Salmonella,* and specifies limits for total bacterial, coliform, mould and yeast counts (Joubert *et al*., 2011). The limits set by these standards are stricter than those of the World Health Organization (WHO) guidelines for herbal teas and infusions prepared with boiling water (Joubert *et al*., 2011).

While industries based on South Africa’s diverse floral kingdom can initially be sustained by harvesting from natural populations, eventually demand will outweigh the supply and sustainability will be compromised (Joubert *et al.*, 2017; Potts, 2017). Transition from wild harvesting to an agriculture should be made in a way that minimises threats to conservation and genetic diversity (Joubert *et al*., 2017; Potts, 2017).

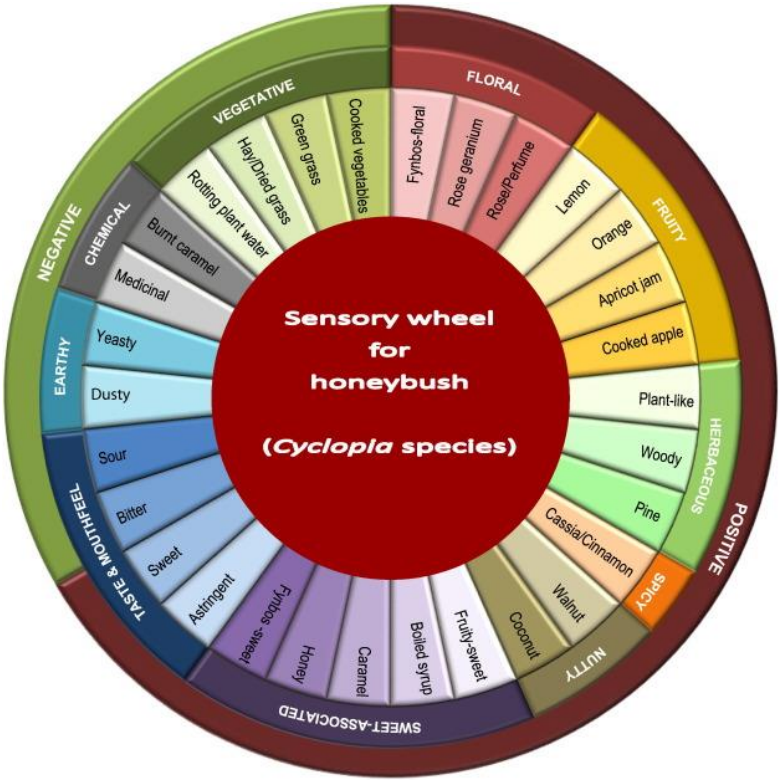

Figure 2.5 Generic sensory wheel for honeybush tea, produced from *Cyclopia* species (Theron *et al*., 2014)

9. PEPPERBARK (*WARBURGIA SALUTARIS*)

Warburgia salutaris is classified as a member of the cinnamon family (*Canellaceae*) and is a sought-after medicinal plant distributed in eastern and southern Africa (Kotina *et al.*, 2014; Leonard & Viljoen, 2015). *W. salutaris* is distributed mainly throughout the savanna woodlands, coastal forest and Afromontane forest regions of KwaZulu-Natal, Limpopo, Mpumalanga, Swaziland, Mozambique and Zimbabwe (Leonard & Viljoen, 2015). The genus *Warburgia* is endemic to the African continent and is considered as 'the panacea of Africa' (Leonard & Viljoen, 2015).

The species epithet "salutaris" means "salutory to health", referring to the initial use of the bark as a health tonic (Van Wyk, 2008; Leonard & Viljoen, 2015). Application as a natural antibiotic in the form of an extract or other by-product has led to its reputation as a popular botanical in traditional medicine (Khumalo *et al*., 2019). It has been used to treat a wide variety of illnesses caused by fungi, bacteria, viruses and insects (Van Wyk, 2008; Khumalo *et al*., 2019).

Medicinal value reported for this tree includes relieving fevers, respiratory problems, stomach ailments, malaria, venereal diseases, cancer and general pain (Kotina *et al.*, 2014; Leonard & Viljoen, 2015). Preparations of the dried stem and root bark are often applied as a

snuff, inhalant, decoction or mixed with porridge (Van Wyk, 2008; Leonard & Viljoen, 2015; Khumalo *et al*., 2019; Van Wyk & Prinsloo, 2019).

Although the powdered bark is the most commonly used part of the plant, the leaves, roots and stalks have also been used for herbal preparations (Figure 2.6) (Leonard & Viljoen, 2015). *W. salutaris* is an evergreen, medium-sized, aromatic tree that grows roughly to 10-20 m (Leonard & Viljoen, 2015; Van Wyk & Prinsloo, 2019). The outer bark is rough and fissured and the inner bark is typically reddish in colour (Figure 2.6) (Van Wyk & Prinsloo, 2019). Yellow, corky lenticels are characteristic of older branches (Van Wyk & Prinsloo, 2019). The leaves of *W. salutaris* are alternately arranged, densely gland-dotted, oblong-shaped, glossy green above and a paler green with a prominent rachis below, and the leaf margins are smooth (Kotina *et al.,* 2014; Van Wyk & Prinsloo, 2019). The leaves are approximately 6 cm long (Van Wyk & Prinsloo, 2019). The flowers of *W. salutaris* are small, greenish-yellow and may be solitary or in 3-flowered cymes in the axils of leaves (Kotina *et al.*, 2014; Van Wyk & Prinsloo, 2019). The flowers are followed by plum-shaped, green to yellowish berries, 20–40 mm in diameter, turning purple when mature (Kotina *et al.*, 2014; Van Wyk & Prinsloo, 2019). The fruits are filled with seeds (Van Wyk & Prinsloo, 2019).

A well-known nickname for *Warburgia salutaris* is the “Pepperbark” tree, descriptive of the fragrant, pungent, peppery taste of the leaves and bark (Leonard & Viljoen, 2015; Van Wyk & Prinsloo, 2019). For this reason, the Pepperbark tree has earned the interest of fragrance, flavour as well as food and beverage industries (Van Wyk & Prinsloo, 2019). Traditionally, fresh or dried leaves are used to impart a pleasant aromatic, peppery taste to various dishes (Leonard & Viljoen, 2015; Van Wyk & Prinsloo, 2019). The leaves have also been used to prepare a pleasant-tasting tea or to stimulate the appetite (Van Wyk & Prinsloo, 2019).

In a review article based on the *Warburgia* genus, Leonard and Viljoen (2015), discusses the some of the compounds responsible for the hot taste in *Warburgia* species. One of the primary hot-tasting compounds isolated from *Warburgia* species was the drimane sesquiterpenoid, polygodial (Leonard & Viljoen, 2015).

In 1981, Kubo and Ganjian isolated the sesquiterpenes, polygodial, warburganal, muzigadial and ugandensidial from extracts of the bark of the East African *Warburgia* species, *Warburgia ugandensis* and *W. stuhlmannii.* These hot compounds are oxidation products of the drimenin skeleton and exhibit powerful antifeedant activity against the armyworms, *Spodoptera exempta* and *S. littoralis* as part of their natural defense mechanism (Kubo, 1995).

The antifungal sesquiterpene dialdehydes, warburganal, muzigadial and polygodial have also been reported as highly active against the fungal species, *Candida utilis, Saccharomyces cerevisiae, Penicillium chryaogenuuif, Hansenula anomala,* and *Sclerotinia lihertiana* (Kubo, 1995). Polygodial and warburganal were subsequently tested against

Candida albicans, and both were found to be highly active effective against this pathogenic fungus (Kubo, 1995). The structurally simple sesquiterpene, polygodial exhibited 2-8 times more antifungal activity than warburganal and muzigadial against the range of fungi tested (Kubo, 1995). The potency of polygodial against the fungi tested is comparable to that of amphotericin B, known as one of the most potent antibiotics against filamentous fungi (Kubo, 1995).

Mashimbye *et al.* (1999) isolated four new drimane sesquiterpenes warburganal, mukaadial, polygodial and isopolygodial in addition to a new lactone drimane sesquiterpene, salutarisolide, from the stem bark of the Pepperbark tree. In 2000, Rabe and van Staden isolated muzigadial from the bark of *W. salutaris* for the first time.

A comparison of bark and leaves showed close similarities in their active terpenoid concentrations and thus the leaves offer a sustainable alternative to the bark (Kotina *et al.*, 2014; Leonard & Viljoen, 2015; Van Wyk & Prinsloo, 2019). Drewes *et al.* (2001) used a novel quantitative ^{1}H NMR procedure to determine the ratio of warburganal and polygodial in the bark and leaves of *W. salutaris*. They concluded that there was no significant difference between the bark- and leaf- content of warburganal and polygodial (Drewes *et al*., 2001). The most abundant drimane sesquiterpene in *W. salutaris* bark was ugandensidial at 0.23%, while muzigadial, polygodial and warburganal were present in concentrations ranging from 0.01% to 0.03% (Drewes *et al*., 2001).

As a consequence of overexploitation and unsustainable harvesting practices, *W. salutaris* has been classified as a Red List species by the International Union for the Conservation of Nature (IUCN) due to excessive harvesting of bark (Leonard & Viljoen, 2015). Plant-part substitution as previously mentioned, growth in protected commercial sites, propagation from soft-wood stem cuttings and *in vitro* micropropagation have all been explored in an attempt to improve long-term sustainability (Kotina *et al.*, 2014; Leonard & Viljoen, 2015).

In Mozambique, the species is listed as ‘vulnerable’, while considered ‘endangered’ in Malawi (Van Wyk & Prinsloo, 2019). *W. salutaris* is ‘extinct in the wild’ in Zimbabwe and ‘critically endangered’ in Swaziland (Van Wyk & Prinsloo, 2019). Research on *W. salutaris* has shown that cultivation is ecologically feasible but that there is a limited availability of seedlings (Van Wyk & Prinsloo, 2019).

Seeds may also be infested with insects and therefore a more successful method of cultivation is to propagate trees from either root suckers, or even better, from stem or tip cuttings (Van Wyk & Prinsloo, 2019).

Steenkamp *et al*. (2005) evaluated the genotoxic activities of 13 South African herbal extracts (Leonard & Viljoen, 2015). As with most of the herbal extracts studied, neither the water nor methanol extracts *of W. salutaris* showed genotoxicity (Leonard & Viljoen, 2015).

Figure 2.6 Commercial products of *Warburgia salutaris*. A and B show bark as it is sold on traditional markets (arrows indicate *Warburgia* amongst other bark products); B showing outer and inner bark; C, commercial bark harvested from young cultivated trees; D, leaf powder; E, dried leaves (Kotina *et al.*, 2014)

10. ANALYSIS

10.1 LC Taste®

LC Taste® is a novel method of analysis that involves the separation of non-volatile compounds in complex food matrices via high temperature liquid chromatography (HTLC) (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c). A blend of non-toxic solvents is used, enabling olfactory-, retronasal-, trigeminal- and taste-perceptions via online or direct sensory evaluation (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c).

The relation of analytical to sensory data is made possible without the need of subsequent steps to remove harmful solvents (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c). As a result, chemical compounds are less susceptible to deterioration or alteration, a common draw-back of conventional high-performance liquid chromatography (HPLC) fractionation using toxic eluents (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c). These methods often require complicated isolation and purification steps to remove harmful solvents before sensory evaluation becomes possible (Reichelt *et al*., 2010a).

A similar protocol to LC Taste®, GC-O, or ‘gas chromatography-olfactometry’, was developed in the 1960s and is a method used for the analysis of volatile flavour compounds, often from complex extracts, ranging from coffee to bread crusts to taints in mineral water (Delahunty *et al*., 2006; Reichelt *et al*., 2010a; Liu *et al*., 2019). The GC-O method combines the use of an olfactometer, an instrument that allows the use of a human nose to act as a sensitive and selective detector of the odour-activity of volatiles after separation via gas chromatography (GC) (Delahunty *et al*., 2006). The technique allows the human assessor to

assign the contribution of the various compounds detected to the overall aroma (Delahunty *et al*., 2006; Liu *et al.*, 2019).

Analysis using GC enables the identification of most of the volatile compounds that comprise an odour (Delahunty *et al*., 2006). Because of the large variance in the perceptibility of odour-active compounds, as well as the absence of a well-established relationship between a compound's structure and its smell, a physical GC detector response will not be a useful representation of a compound's odour activity (Delahunty *et al*., 2006). Research has established that the human nose is usually a more sensitive tool to evaluate odour-active compounds than physical detectors (Delahunty *et al*., 2006). Consequently, GC-O employs a human tester to smell the resolved volatiles as they elute from a GC column, to establish whether components are above their specific absolute threshold, and to determine their intensity and odour quality at a given concentration (Delahunty *et al*., 2006; Song & Liu, 2018; Liu *et al*., 2019).

The GC-O system involves the injection of a food extract or distillate into a GC, fitted with up to four odour ports in the place of, or to supplement a conventional detector, gas chromatography-mass spectrometry (GC-MS) or gas chromatography-flame ionisation detection (GC-FID) (Song & Liu, 2018). The eluate is divided between the mass spectral detector and olfactory port(s) where 'sniffers' are seated at the 'nose cones', typically a glass cone or Teflon sleeve, to document the smell(s) detected in the humidified air stream (Delahunty *et al*., 2006). MS enables simultaneous identification of odour-active compounds (Delahunty *et al*., 2006; Liu *et al.*, 2019). Another variation of detection is to use an in-line, non-destructive detector, such as a thermal conductivity detector (TCD) photo-ionisation detector (PID) (Delahunty *et al.*, 2006).

Driven by the need to discover key compounds contributing to the taste of food, knowledge of the research area 'sensomics' has recently expanded, with researchers delving into the so-called 'sensometabolome' of products, defined as the composition of the group of non-volatile, sensory active key molecules responsible for the sensory perception of tastes and flavours (Mittermeier *et al.*, 2018).

Using the same principle as GC-O, LC Taste® involves initial separation or fractionation of non-volatiles from food extracts via reverse phase-high temperature liquid chromatography (RP-HTLC), coupled to taste dilution analysis (TDA) to identify taste-active fractions (Reichelt *et al*., 2010a; Reichelt *et al*., 2010b). Correlation of this data with analytical detection, for example, liquid chromatography-mass spectrometry (LC-MS) or liquid chromatography–diode-array detection (LC-DAD), allows structural elucidation and quantification, if required (Reichelt *et al.,* 2010b; Mittermeier *et al.*, 2018).

Several LC methods have been developed for the isolation of non-volatile compounds from complex natural products, for instance preparative HPLC (pHPLC), or various liquid-

liquid partition chromatography techniques, for example high speed counter-current chromatography (HSCCC) or fast centrifugal partition chromatography (FCPC) (Reichelt *et al*., 2010b). A challenge faced with these methods is that they require the use of toxic solvents such as methanol or acetonitrile, which cannot be directly used for human taste evaluation (Reichelt *et al*., 2010b; Yabré *et al*., 2018). Cost-, labour- and time-intensive procedures for the isolation of single compounds or interesting fractions is necessary before sensory testing becomes safe for the tasting panel (Reichelt *et al*., 2010b).

LC Taste® was designed to address the shortcomings of conventional separations of food mixtures, providing a more rapid and efficient alternative (Reichelt *et al*., 2010a). The technique was patented by Symrise in 2005, with the breakthrough being that high performance liquid chromatography now be used for the separation of non-volatile compounds from complex solutions, while performing simultaneous sensory evaluations (Symrise, 2005).

LC Taste® utilises water as the primary mobile phase component, although other non-toxic compounds such as physiologically tolerable alcohols, salts, acids, buffers, oils or fats may be incorporated (Reichelt *et al*., 2010). Elevated temperatures are often used in combination with ethanol mobile phases to reduce mobile phase viscosity to circumvent pressure constraints (Reichelt *et al*., 2010).

Possible applications of LC Taste® include the identification of undesirable off-notes as well as desirable taste active compounds or taste modulators in complex food matrices (Reichelt *et al.*, 2010a; Mittermeier *et al.*, 2018). While conventional TDA is executed by consecutively diluting a previously isolated compound, a more rapid method of coupled taste dilution analysis was developed for LC Taste®, thereby creating valuable screening protocol without previous isolation of compounds (Reichelt *et al.,* 2010b). Once the real-time analysis is complete, sensory evaluation by a specifically trained panel enables the detection of relevant taste-active or -modifying compounds (Reichelt *et al.*, 2010b; Mittermeier *et al.*, 2018).

The first published reference to the protocol was in 2010, when Reichelt and her team successfully implemented LC Taste® in a study of flavour modulators in botanical extracts (Reichelt *et al.* 2010a; Reichelt *et al.,* 2010b, Reichelt *et al*., 2010c). The difficulty with identifying flavour-modifying agents is that ideal modulators themselves show very weak, or even no intrinsic flavour, since their role is to mask certain bitter- or off-notes or enhance sweetness or saltiness, rather than alter the flavour profile of the given food product (Reichelt *et al*., 2010c; Wu *et al*., 2016). While the detection of sweet and bitter compounds is relatively simple to detect through tasting, different test bases are required to detect the effect of modulators (Reichelt *et al*., 2010c). This means without LC Taste®, time-intensive isolation of large quantities of analytes are required for tests to be performed using a variety of tastants (Reichelt *et al*., 2010c). Common test bases include sucrose as well as bitter-tasting test-

bases such as caffeine, hydrophobic peptides and catechins, for instance (Reichelt *et al*., 2010c).

The LC Taste® methodology was used to analyse known flavour modulators, namely homoeriodictyol, sterubin, hesperitin and lactisol, as well as simple mixtures of these substances (Reichelt *et al*., 2010c). To evaluate the potential of the protocol in more realistic matrices, it was also applied to botanical extracts from Yerba Santa (*Eriodictyon californicum*) and honeybush tea (*Cyclopia intermedia*), known to contain the flavour modulators, homoeriodictyol, steburin and hesperitin (Reichelt *et al*., 2010c).

After LC Taste® fractionation, sensory analysis was conducted in a blind paired-comparison test together with a blank sample containing water that was fractionated under identical conditions to the test sample, resulting in samples with the same ethanol concentration (Reichelt *et al*., 2010c). The water was also blended with either sucrose or caffeine solutions in the same ratio (Reichelt *et al*., 2010c). Panellists were asked to compare the samples and to specify the sweeter or more bitter sample, depending on the test solution (Reichelt *et al*., 2010c).

Although the HTLC method could still be improved by column selection for optimal peak separation, LC Taste® enabled an accelerated primary evaluation of single fractions as well as the selection of taste-active fractions for further sensory analysis (Reichelt *et al*., 2010c). Since compounds can be tested at their naturally occurring concentrations, it also provides a good indication of the contribution of a flavour modulating fraction to the overall flavour of a sample (Reichelt *et al*., 2010c).

In a similar study, LC Taste® was used to identify taste modulators in Yerba Santa and traditional African teas, specifically honeybush and unfermented Rooibos tea (*Aspalathus linearis*) (Reichelt *et al*., 2010b). Homoeriodictyol and hesperetin were identified as principal taste modulators in Yerba Santa and honeybush, whereas, in contrast to previous literature findings, no taste activity was detected for the supposedly sweet compound, aspalathin in rooibos tea (Reichelt *et al*., 2010b).

Obtained fractions are suitable for immediate sensory evaluation, although in this study, the fractions were collected for comparative purposes (Reichelt *et al*., 2010b). For coupled taste dilution analysis, extracts were fractionated and collected peak-wise or after determined time intervals before being presented to the tasting panel (Reichelt *et al*., 2010b). Dilution steps involved repeated 1:1 dilution of the stock solution with the appropriate solvent, either ethanol, water, or a combination of the two (Reichelt *et al*., 2010b). The samples were presented in decreasing order of strength for sensory evaluation, until no taste was detected by the tasters to determine the taste dilution (TD) factor, the highest dilution factor at which a taste can be detected (Reichelt *et al*., 2010b; Mittermeier *et al*., 2018). Duo-tests were

performed to evaluate taste modulating compounds for sweetness and bitterness (Reichelt *et al*., 2010b).

In 2012, Reichelt and her team investigated another indigenous South African tea, namely bush tea or *Athrixia phylicoides*, for potential taste modulators (Reichelt *et al.,* 2012). Using HTLC-coupled sensory-guided analysis, another description of LC Taste®, a methanolic extract of the tea was fractionated and was evaluated sensorially to find a fraction that showed bitter enhancing effects on a caffeine solution (Reichelt *et al*., 2012). Two separate compounds were identified in the fraction subsequently by using conventional fractionation and preparative HPLC followed by structural elucidation using nuclear magnetic resonance (NMR) and LC-NMR, which led to the uncovering that when the individual compounds isolated from the fraction were tasted in caffeine solutions separately, quercetin-3′-*O*-glycoside enhanced bitterness, however methoxyquercetin-3′-*O*-glycoside was not perceived as a taste-modulator (Reichelt *et al*., 2012). The results suggested that the bitter enhancing effect of the LC Taste® fraction might also be due to a synergistic effect between the compounds present in the fraction (Reichelt *et al*., 2012).

The most recent application of LC Taste® was in 2014, when Reichelt teamed up with another group of researchers to investigate the flavour potential of the roots of the woody climber, *Mondia whitei* (Asclepiadaceae) (Reichelt *et al.*, 2014). The root of this African plant was traditionally consumed due to its vanilla-like flavour and sweet aftertaste, although it is also known for its bitter taste (Reichelt *et al.*, 2014). Peak-wise LC Taste® fractionation and sensory evaluation, followed by LC-MS and GC-MS, led to the uncovering of the vanillin isomer, 2-hydroxy-4-methoxy-benzaldehyde (HMBA), as a sweetness-enhancing compound (Reichelt *et al.*, 2014). The taste-modulation property was best detected at a concentration of 0.5 $mg.L^{-1}$; above this concentration, the intrinsic, coumarin-like flavour was perceived as too strong and overwhelmed the trained panellists (Reichelt *et al.*, 2014).

To determine whether the sweetness enhancement was due to synergistic flavour effects, the tests were repeated using nose clamps, finding that the perceived effect was mainly owed to a “sweet” aroma impression, similar to what is known for the related compound, vanillin. In a simple beverage application, it was shown that HMBA was able to restore the loss of sweetness in a 20% sugar reduced beverage (Reichelt *et al.*, 2014).

Although very limited applications of LC Taste®-based investigations have been published to date, the relevant researchers have reported the protocol as a valuable flavour screening tool, providing an accelerated approach to the identification of single fractions with potentially interesting taste-active components for further analysis (Reichelt *et al*., 2010b; Reichelt *et al*., 2012).

10.2 High Performance Liquid Chromatography (HPLC)

Liquid chromatography is generally more expensive and generates more waste than gas chromatography, however, it is required when analytes are not sufficiently volatile for GC (Harris, 2010). HPLC is a high-resolution separation technique, based on forcing a solvent containing analytes through closed columns, packed with small particles (De Villiers *et al*., 2009; Harris, 2010). Since the efficiency of a packed column is inversely related to particle size, typical particle sizes used in HPLC are as small as 1.7 to 5 µm, with smaller particles resulting in sharper peaks or shortened analysis, associated with reduction in column length, if resolution is maintained (De Villiers *et al*., 2009).

The draw-back of reducing particle size is the build-up of back pressure, resulting in resistance to solvent flow (Harris, 2010). Consequently, a certain pressure is needed to drive the solvent though the column – the pressure is proportional to flow rate and column length and inversely proportional to the square of the column radius and square of particle size (Harris, 2010).

10.3 High Temperature Liquid Chromatography (HTLC)

High-temperature liquid chromatography refers to separations conducted at temperatures above ambient temperature, typically from 40°C to 200°C, with a liquid mobile phase (Heinisch & Rocca, 2009). HTLC is a valuable technique in RP-HPLC since manipulation of column temperature can facilitate the reduction of analysis time, modify analyte retention, control selectivity, change efficiency, reduce the amount of organic solvent required or improve detection sensitivity (Yang, 2008; Heinisch & Rocca, 2009). The practical limitations of elevated temperatures on column and analyte stability limits the application of HTLC, especially when polar or ionised compounds are being separated (Yang, 2008; Heinisch & Rocca, 2009).

In most cases, the retention of solutes decreases with increasing temperature, especially when dealing with non-polar analytes (McNeff *et al*., 2007; Teutenberg, 2009). This can be illustrated by linear van't Hoff plots in which the inverse of the absolute temperature is plotted against the natural logarithm of the retention factor (McNeff *et al*., 2007). Using phenol as an example, Gritti and Guiochon studied the effect of temperature on adsorption and retention behaviour using a silica-based C-18 Reverse Phase column and a mobile phase comprised of methanol or acetonitrile (McNeff *et al*., 2007). The study suggested that non-linear van't Hoff plots could be the result of factors such adsorption isotherms, surface heterogeneity, saturation capacities, and equilibrium constants (McNeff *et al*., 2007). Further research into deviations from the linearity of van't Hoff plots have pointed to desorption kinetics of different functional groups differing from one another, dual retention mechanisms as well as possible undesired temperature-dependent changes to mobile phase, resulting in changes

in eluent strength (McNeff *et al*., 2007). In contrast to small solutes, for example, proteins and polypeptides, could in fact show an increase in retention with a temperature increase (McNeff *et al*., 2007).

The effect of temperature on mobile phase pH and analyte pKa's, and subsequently on retention behaviour, cannot be ignored in HTLC (Teutenberg, 2009). In a review article on HTLC, Teutenberg advises experimental proof of linearity over a selected temperature range (Teutenberg, 2009).

The reduced viscosity of the mobile phase, coupled to increased column temperatures, lowers the back pressure experienced in the HTLC column – this facilitates not only faster linear flow rates and analysis times, but also the use of longer columns with smaller particle packing which greatly enhances separation efficiency (Marin *et al*., 2004)

The van Deemter equation (equation 1) has been used to unravel the effect of temperature on RP-LC column efficiency (McNeff *et al*., 2007):

$$H = A + B/u + Cu \qquad (1)$$

Where H represents the plate height or Height Equivalent of a Theoretical Plate (HETP) and u represents mobile phase linear velocity (equation 1) (McNeff *et al*., 2007; Gritti & Guiochen, 2012). Band broadening is accounted for by the *A*-term, known as the Eddy diffusion coefficient, reflecting the effect of column packing uniformity, the *B*-term which accounts for longitudinal diffusion and the *C*-term, which represents the resistance to mass transfer between the mobile phase and stationary phase (equation 1) (McNeff *et al.*, 2007; Teutenberg, 2009; Gritti & Guiochen, 2012). Although it is often assumed that the *A*-term is temperature-independent, research has shown that eluent temperatures could affect the laminar flow and lateral mixing of molecules among the multiple paths in a column (equation 1) (Teutenberg, 2009). The *B*-term and *C*-term both rely on temperature, with the *B*-term directly proportional to and the *C*-term inversely proportional to the diffusion coefficient of the analyte in the mobile phase (equation 1) (Teutenberg, 2009). The diffusion coefficient is in turn, directly proportional to temperature and inversely proportional to viscosity (Teutenberg, 2009). In other words, increasing the temperature will enhance the diffusion of solutes in the mobile and stationary phases (Teutenberg, 2009).

Results by Vanhoenacker and Sandra proved that by proper mobile phase preheating, radial temperature gradients can be avoided, rendering a plate number that is largely independent of the analysis temperature (Teutenberg, 2009). This is associated with an accompanied shift of the van Deemter curve, moving the minimum H to a higher linear velocity (equation 1) (Yang, 2008; Teutenberg, 2009). In addition, the sharp increase of H as a function of flow rates greater than the optimum linear velocity (u_{opt}) is less distinct at elevated

temperatures (equation 1) (Yang, 2008; Teutenberg, 2009). Systems operating at mobile phase flow rate greater than the optimum flow rate will benefit from the effect of temperature on separation efficiency (Yang, 2008; Teutenberg, 2009).

The effect of temperature on selectivity is also a widely recognised phenomenon (Dolan, 2002; Heinisch & Rocca, 2009). Researchers have listed various situations where an alteration in selectivity with temperature is advantageous (Dolan, 2002; Heinisch & Rocca, 2009). Examples when this is the case are when the relative retention of two solutes is affected by temperature dependent changes in the conformation of the stationary phase, when the size or shape of two molecules differ, resulting in different entropies, when two molecules have different functional groups with a different temperature dependence for the retention or when an ionisable solute is partially ionised and the molecule exists in both ionised and neutral forms (Dolan, 2002; Heinisch & Rocca, 2009).

Changes in selectivity with temperature has been recorded for certain polymers on various stationary phases (Heinisch & Rocca, 2009). Irregular temperature-dependent behaviours, such as increased retention with temperature could be assigned to different conformational states of the molecules dominant at different temperatures (Heinisch & Rocca, 2009).

10.3.1 Mobile phases in Reverse Phase (RP)-HPLC and -HTLC

HPLC methods are mostly based on the reversed-phase mode, consisting of a non-polar or weakly polar stationary phase and a more polar mobile phase (Yabré *et al.,* 2018). In RP-chromatography, the eluent strength of the solvent (and elution speed) is increased by making it more like the stationary phase, in other words, less polar (Harris, 2010). Mixtures of organic solvents, acetonitrile, methanol and tetrahydrofuran with an aqueous buffer (water), supply a series of dipolar and hydrogen-bonding interactions with analytes, enabling the separation of many compounds in RP-chromatography (Vanhoenacker & Sandra, 2005; Yabré *et al*., 2018). The preferred solvent choice is a mixture of acetonitrile and water due to the low viscosity and low UV (Ultraviolet) wavelength cut-off of acetonitrile (190 nm) (Yabré *et al*., 2018). Next in line is methanol (UV cut-off 205 nm) and lastly tetrahydrofuran because of its significant UV absorption below 250 nm, its slow column equilibration as well as its chemical reactivity that may result in solvent and column degradation (Harris, 2010).

The consumption of organic solvents can be reduced or even eliminated in HTLC while maintaining retention, thereby saving costs and reducing environmental impacts related to disposal of solvents (Yabré *et al*., 2018). At ambient temperature conditions, the viscosity of ethanol is too high for it to be used as a mobile phase component. At higher temperatures, ethanol's viscosity and thus column back-pressure is reduced, making it a viable, greener alternative to solvents such as methanol and acetonitrile (Yabré *et al*., 2018). It is essential to

consider the thermal stability of the stationary phase in cases of HTLC (Vanhoenacker & Sandra, 2005). The maximum temperature at which silica-based columns should be operated should not exceed 60°C, unless a more temperature resistant column is applied, in which case efficiency might be compromised (Yang, 2008). This topic is addressed in more detail further on.

In cases where organic solvents are eliminated, a pure water eluent at elevated temperature offers an alternative with plenty of advantages (Yabré *et al*., 2018). Despite being environmentally friendly, it is readily available, highly affordable, non-toxic and non-flammable (Yabré *et al*., 2018). Since water has a low UV cut-off (190 nm), it enables the detection of weak chromophores (Yabré *et al*., 2018). Detectors not typically used with LC, such as universal FID can be used because the lack of FID response of water, providing a means of detection on UV-transparent compounds (McNeff *et al*., 2007). Furthermore, MS detectors can be used with heated water to improve ionisation efficiency and signal-to-noise ratio (S/N) of some analytes (Yabré *et al*., 2018). It has, however been reported that the sensitivity of ESI (electrospray-ionisation)-MS detection is likely to decline as the percentage of organic solvent decreases (Heinisch & Rocca, 2009).

The application of a 100% aqueous mobile phase in RP-HPLC is limited because of the high polarity of water, resulting in insufficient elution strength to separate non-polar analytes at ambient temperature (Marin *et al*., 2004; Yabré *et al*., 2018). Hydrogen-bonding in water is reduced at elevated temperatures, resulting in decreased polarity and increased eluent strength in RP-HTLC (Marin *et al*., 2004). Consequentially, the use of pure water generally becomes viable at temperatures above 100°C (Heinisch & Rocca, 2009). Techniques applied using pure water solvents include superheated water chromatography (SHWC), using elevated temperatures, or subcritical water chromatography (SWC) when the temperatures used are lower than the critical temperature of water (374°C) (Yabré *et al*., 2018). Studies have shown that the solvent strength of pure water at 150°C is similar to a 50:50 ($v.v^{-1}$) mixture of water–methanol at ambient temperature (Yabré *et al*., 2018).

The convenience of this changing polarity is that a single solvent can be used to replace a binary solvent gradient separation by making use of temperature programming (Marin *et al*., 2004). The difficulty of a separation that relies solely on a temperature gradient to increase eluent strength, is the need for a large temperature range, often with a steep temperature-ramp to mimic the effect of a solvent gradient (Heinisch & Rocca, 2009). Limitations of temperature-programming are discussed in more detail further on.

Phase-collapse or phase de-wetting is another problem that arises from the use of pure water as a solvent (Yabré *et al*., 2018). It occurs in alkyl bonded phases such as C-8 or C-18, and may lead to problems including retention loss, peak tailing, non-reproducible retention times, and gradient regeneration delays (Yabré *et al*., 2018). Ways to combat this

phenomenon include to use stationary phases specifically designed to accommodate the use of a water mobile phase (Yabré *et al*., 2018). Design features include the possibility of polar end-capping or polar embedded groups (Yabré *et al*., 2018). High temperature water is an aggressive eluent and temperature-resistant stationary phases such as polymeric phases or zirconia-based materials are required to prevent the accelerated dissolution of silica initiated by the reactive properties of water (Vanhoenacker & Sandra, 2005; Yabré *et al*., 2018). Unfortunately, there is limited availability of thermally stable stationary phases suitable for superheated water (Heinisch & Rocca, 2009).

An additional concern of SHWC includes the insolubility of hydrophobic analytes in water, in which case a stronger injection solvent could be put into practice, but which may result in peak distortion (Yabré *et al*., 2018).

10.3.2 Columns and analyte stability of HTLC

Although HTLC holds many advantages, it also has its limitations (Yabré *et al*., 2018). Thermal stability of stationary phases and analytes present two challenges (Vanhoenacker & Sandra, 2005; Yabré *et al*., 2018).

Several silica-bonded packings have shown good stability to temperatures around 100°C in the long run, however many of them are not very temperature-stable (Vanhoeacker, 2005; Yang, 2008). Zirconia- and other metal oxide-based columns are slightly more heat-resistant and can generally withstand operational temperatures as high as 200°C (Vanhoenacker & Sandra, 2005; McNeff *et al*., 2007). Polymer stationary phases are, however, the most stable LC packings, and can be used reliably at temperatures as high as 150°C or greater, although some efficiency is compromised in these columns (Yang, 2008). Additional stationary phases that have been tested for HTLC suitability are carbon, monolithic, and temperature-responsive packings (Yang, 2008).

Because of repeated exposure to high temperatures, packing materials in stationary phases suffer greater strain than analytes, owing to the shortened analysis times, characteristic of HTLC (Yang, 2008). Most analytes are stable on the normal time scale of an HTLC run and therefore analyte degradation is less concerning than column stability (Yang, 2008).

The thermal degradation of analytes can be quantitatively evaluated by calculating the Damköhler number (*Da*) (de Villiers *et al.*, 2009). This value increases as the time that the analyte resides within the column increases, thus *Da* holds an inversely proportional relation to the mobile phase linear velocity (de Villiers *et al*., 2009). Thompson and Carr suggested that the susceptibility of analytes to HTLC analysis is only of concern in cases where $0.1 < Da < 50$, i.e. when thermal degradation occurs on a similar time scale to that of the analysis. In

these instances, on-column degradation may compromise chromatographic efficiency leading to invalid quantitative data (de Villiers *et al*., 2009).

10.3.3 Isocratic and Gradient Elution and Temperature Programming

In an isocratic elution, a single solvent, or constant solvent combination, is employed (Harris, 2010). Gradient elution involves a constant change in the solvent composition, gradually increasing the eluent strength to increase the speed of elution of more strongly retained analytes (Harris, 2010).

Temperature-programmed HPLC is a more recently proposed method to replace traditional solvent gradient elution and involves a change in the column temperature as a function of time (Heinisch & Rocca, 2009). Temperature programming is ideal in separations performed with micro- or nano-columns, when a gradient elution is challenging to operate at very low flow-rates (Vanhoenacker & Sandra, 2005; Heinisch & Rocca, 2009). In the instance when superheated water serves as the mobile phase, a temperature gradient is the only way of increasing the eluent strength (Heinisch & Rocca, 2009; Yabré *et al*., 2018). Temperature-programming could also replace gradient elution in systems with large dwell volumes, eliminating the need for large isocratic holds (Heinisch & Rocca, 2009).

Due to current limitations of instrumentation, temperature-programming is often used in combination with gradient elution (Heinisch & Rocca, 2009). This is because the technique requires a wide range of temperatures, often with a very steep temperature ramp, to be successful (Heinisch & Rocca, 2009). Because of slow heat transfer in liquids, as well as the massive amount of steel to be heated in the case of conventional HPLC columns, capillary columns with narrow diameters are recommended to improve heat transfer (Vanhoenacker & Sandra, 2005; Heinisch & Rocca, 2009).

10.3.4 Thermal mismatch

When solvent that is not properly preheated flows through a heated column, the innermost packing is cooled (Clark, 2004). Solvent along the column wall is heated faster than the central solvent, resulting in a radial viscosity gradient in the column (Clark, 2004). The solvent moves faster along the periphery where the viscosity is lower than at the centre (Clark, 2004). Similarly, a longitudinal gradient is set up between the column inlet and outlet if the entering mobile phase is below the set column temperature (Heinisch & Rocca, 2009). This phenomenon is known as thermal mismatch (Clark, 2004). While the radial thermal gradient results in significant peak broadening, loss of resolution and even peak distortion, the longitudinal gradient creates a gradient of retention factors across the column but does not significantly affect the column plate number (Clark, 2004; Heidorn, 2019; Heinisch & Rocca, 2009). Frictional heating between the mobile phase and the column bed in combination with

poor heat dissipation in packed columns is an additional source of longitudinal and radial thermal gradients across the column (Heinisch & Rocca, 2009). The significance of this effect increases linearly with operating pressure (Heinisch & Rocca, 2009).

Researchers have discovered that the key to avoiding the effects of thermal mismatch and to ensure successful extended-range HPLC is to incorporate efficient mobile phase preheating (Clark, 2004). The effect of pre-heating on analyte degradation is outweighed by the positive effects of minimising peak broadening and distortion (Clark, 2004). It has been found that the temperature difference between the incoming mobile phase and the column temperature should not exceed ±5°C to maintain good column performance (Heinisch & Rocca, 2009).

The effect of insufficient preheating has been demonstrated using the separation of alkylbenzenes at 150°C as an example (Heinisch & Rocca, 2009). A forced air circulating oven and a flow rate of 4 $mL.min^{-1}$ illustrated dramatic peak distortion on a 1 m-long column, with the most pronounced effect on compounds with greater retention factors (Heinisch & Rocca, 2009). A substantial increase in the retention of more retained solutes was observed (Heinisch & Rocca, 2009).

The concern of loss of efficiency associated with long preheating devices (i.e. extra-column band broadening) has been overcome by commercially available fast-response preheaters that use small volumes of mobile phase to achieve the necessary heat transfer (McNeff *et al*., 2007). Currently available design options for mobile phase preheating include longer lengths of tubing connecting the injector to the column, shorter lengths of tubing embedded in heater blocks that make use of enhanced heat transfer via metal-metal contact or counter current heat exchange of energy from the outlet of the column to the entering mobile phase (Heinisch & Rocca, 2009).

The loss of separation efficiency due to radial temperature gradient can be reduced by decreasing the column diameter (Heidorn, 2019). Narrow-bore columns (2.1 mm I.D. (internal diameter)) rather than conventional ones (4.6 mm I.D.) provide greater control of the mobile phase and column temperature due to the lower thermal mass and enhanced thermal response (Greibokk & Anderson, 2003; Heinisch & Rocca, 2009).

Thermal output is regulated by a thermocouple immediately downstream from the heater (Heinisch & Rocca, 2009). This form of active feedback heating requires shorter tubing (Heinisch & Rocca, 2009).

Similarly, thermal mismatch can be prevented in detection by cooling the mobile phase (Heinisch & Rocca, 2009). As the vapour pressure of the mobile phase increases with increasing temperature, for example via frictional heating or elevated temperature LC, especially when performing separations at temperatures above 60°C, organic solvents may reach their boiling temperature (Heidorn, 2019). Boiling of the eluent is often supressed by

high pressures in the column but once it elutes from the column, vapour bubbles in the detector may lead to increased baseline noise, ghost peaks and column bleeding (Heidorn, 2019).

In addition, when the separation is run at temperatures greater than 60°C, post-column mobile phase cooling may be required to prevent damage to heat-sensitive detector flow cells (Heinisch & Rocca, 2009). Mobile phase cooling is unnecessary at column temperatures below 100°C if the detector cell was not exposed to high back-pressure conditions (Heinisch & Rocca, 2009).

In the case of low-temperature or sub-ambient separations it may be beneficial to increase the temperature of the mobile phase prior to prevent moisture condensation in an optical detector to prevent moisture (Heidorn, 2019).

In other words, a post-column heat exchanger offering the possibility to heat or cool the mobile phase before detection is important (Heidorn, 2019).

10.3.5 HTLC instrumentation

To successfully implement high temperatures in LC, a dedicated heating system should be implemented to enable independent control of the eluent temperature and the column temperature (Teutenberg, 2009). It should consist of a low volume preheater, capable of heating the mobile phase to the desired temperature, a thermally controlled column compartment, and a heat exchanger to adjust the temperature of the mobile phase before it reaches the detector (McNeff *et al.*, 2007). Different heating ovens are available for isothermal high temperature operation as well as for temperature programming (Fallas *et al.,* 2009; Teutenberg, 2009).

Column temperature can be controlled by block-heaters, air-baths with either still-air or forced, circulating air, liquid (water or oil) baths or via direct heating of the column through electrical resistance in contact with the column (Fallas *et al.,* 2009; Teutenberg, 2009).

With block-heaters, the close contact between the column and the oven allows very efficient and rapid heat transfer (Teutenberg, 2009). The column wall therefore maintained at the set column compartment temperature (Heidorn, 2019). The column wall acts as a heat-exchanger when mobile phase enters the column at a different temperature to the constant wall temperature, tempering the mobile phase temperature in the wall region (Heidorn, 2019). The behaviour of a block heater is classified as quasi-isothermal (Heidorn, 2019).

A still-air oven results in less efficient heat transfer through the column wall and the column therefore gradually reaches its desired temperature (Fallas *et al.,* 2009; Heidorn, 2019). The column wall temperature is subject to change when mobile phase enters at a different temperature seeing that the heat exchange efficiency of still air thermostats are not able to maintain a constant column wall temperature (Fallas *et al.,* 2009; Heidorn, 2019). This

behaviour is classified as quasi-adiabatic and requires mobile phase pre-heating (Lambert & Felinger, 2018; Heidorn, 2019).

While forced air thermostats display more isothermal than adiabatic behaviour, block-heaters are far more efficient heat exchangers (Fallas *et al.,* 2009; Lambert & Felinger, 2018). Heat exchange between the oven and column wall is enhanced by forced air thus when the mobile phase has a different temperature than the column wall, the column wall temperature is slightly adjusted but remains close to the set column compartment temperature (Lambert & Felinger, 2018).

With an isothermal column compartment (i.e. block-heaters, water baths and forced-air), a radial temperature gradient arises in the column if the mobile phase and column compartment temperature are not equal. Radial temperature gradients dominate in this case (Heinich & Rocca, 2009; Lambert & Felinger, 2018). In an adiabatic column compartment (i.e. still air), an axial gradient dominates (Fallas *et al.,* 2009; Heinisch & Rocca, 2009). Depending on the mobile phase flow rate, the column temperature could change to the temperature of the mobile phase rather than the set column temperature (Heidorn, 2019). Consequentially, without adequate mobile phase preheating, a still air thermostat could result in retention time shifts and changes in whereas peak broadening and distortion could occur in block-heaters, water baths and forced air ovens (Fallas *et al.,* 2009; Lambert & Felinger, 2018).

Temperature programming presents additional instrumental challenges (Heinisch & Rocca, 2009). Certain column heaters are eliminated since ovens must rapidly return to the initial temperature of the temperature program (Heinisch & Rocca, 2009). Forced air ovens are a possible candidate when used in conjunction with capillary columns (Heinisch & Rocca, 2009).

11. SENSORY EVALUATION

Sensory evaluation techniques have been shown to play an integral part in the development, production and quality maintenance of food products (Stepanova *et al*., 2012; Van Wyk & Gorelik, 2017). While understanding the nutritional and functional benefits of healthy ingredients is vital to developing a healthy, trendy product, if a product does not meet the sensory expectations of consumers, regardless of its health properties, it is unlikely to sell (Stepanova *et al*., 2012; Van Wyk & Gorelik, 2017).

Sensory evaluation is concerned with various perceptions of humans in response to a physical stimulus, for the purpose of this study, to food (Low & Zhou, 2018). It concentrates on ‘product understanding’, which are objective reactions to sensory aspects of a product, as well as the subjective responses, termed ‘consumer understanding’ (Civille & Oftedal, 2012). Understanding the response of an individual to a product requires linking product and consumer understanding (Forde, 2016).

Sensory studies can be broadly divided into three categories: flavour analysis, which is the relation of chemical compounds to tastes and odours of a food product as perceived by consumers; sensory profiling, which is the determination of sensory attributes such as sweetness, chewiness, and other similar attributes; and lastly, hedonic testing, in which consumer acceptance or preference is evaluated (Yu *et al.*, 2018).

Objective measurements of product sensation or understanding can be either discriminative or descriptive (Prescott *et al.*, 2014). Methods of discrimination involve forced choice tests such as triangle tests or duo-trio tests and are used to determine whether two or more products differ significantly from one another (Prescott *et al.*, 2014). Descriptive sensory analysis evaluates both qualitative and quantitative properties of a product and therefore requires a well-trained panel (Granitto *et al*., 2008; Theron *et al.*, 2014). Qualitative parameters, known as attributes, include specific aspects of appearance, aroma, flavour or texture that can be quantitatively evaluated based on intensity (Granitto *et al*., 2008). 'Aroma' can be defined as the aromatics, perceived orthonasally, for example, as detected via GC-O; 'flavour' as a retronasal perception, closely associated with taste; 'taste' as simply the modality of taste and 'mouthfeel' as the tactile perceptions in the oral cavity (Prescott *et al.*, 2014; Theron *et al.*, 2014; Forde, 2016). A scale may be used to assign values related to the perceived intensity of an attribute (Granitto *et al*., 2008; Civille & Oftedal, 2012; Prescott *et al.*, 2014).

Food producers acknowledge that liking is not enough to drive consumption and that habitual acceptance and consumption patterns are determined by combinations of sensory and post-ingestive food properties (Forde, 2016). Consumer understanding techniques measure the personal reaction of consumers, such as consumer acceptance or preference (Carpenter *et al.,* 2012; Civille & Oftedal, 2012; Forde, 2016). Consumer testing can be either qualitative or quantitative (Civille & Oftedal, 2012; Prescott *et al.*, 2014). Qualitative consumer tests generate verbal information, using open-ended questions and ideally receives in-depth answers to understand why a product is liked or disliked or gain knowledge on any emotional ties to sensory properties (Carpenter *et al.,* 2012; Civille & Oftedal, 2012; Prescott *et al.*, 2014). Quantitative tests involve questions to which the response can be given on a hedonic scale (Prescott *et al.,* 2014). They require large numbers of participants due to the large variation in consumer preference (Carpenter *et al.,* 2012; Civille & Oftedal, 2012; Prescott *et al.*, 2014; Forde, 2016).

Consumer understanding measures the consumer's perception of product attributes, based on an array of expectations (Prescott *et al.*, 2014). Certain product performance benefits are important are not only for brand identity and product quality but also setting up consumer expectation (Prescott *et al.,* 2014). A consumer will have different expectations of a healthy product compared to one marketed as an indulgent product, for example.

In order to obtain valid, realistic results that are reproducible, sensory studies must be carefully designed to strike a balance between highly controlled testing environments and the many sources of variation associated with normal consumption (Carpenter *et al.,* 2012; Prescott *et al.*, 2014). When planning a sensory test, the so-called '5 S's' should always be thoughtfully considered: subjects, site, samples, the sensory method and statistical analysis (Civille & Oftedal, 2012).

Samples that are representative of the product should be tested as intended to be consumed by either a trained panel or a panel that is representative of the targeted consumers, for example (Carpenter *et al.,* 2012; Civille & Oftedal, 2012; Prescott *et al.*, 2014). The sensory method is selected based on the objective and the study (Carpenter *et al.,* 2012; Yu *et al.*, 2018).

The data obtained is processed via statistical analysis to supply information that can be easily interpreted (de Pelsmaeker *et al*., 2019). Analysis of variance and chi-squared are fundamental univariate techniques that are useful to determine simple differences and to make recommendations for quantitative consumer testing (Civille & Oftedal, 2012).

As sample sizes increase, there is a demand for multivariate data analysis techniques to condense the data (Prescott *et al.*, 2014; Yu *et al.*, 2018). In descriptive evaluation, for instance, when many attributes are assigned to various products, principal component analysis (PCA) or factor analysis (FA) can be used to group attributes into relevant dimensions that summarise the variability between the samples (Civille & Oftedal, 2012; Prescott *et al.*, 2014). Cluster analysis is another technique that enables the identification of preference segments (Civille & Oftedal, 2012; Prescott *et al.*, 2014).

Regression techniques can be applied to discover which product attributes are the ones driving consumer acceptance or the perception of benefits (Prescott *et al.*, 2014; Yu *et al.,* 2018; de Pelsmaker *et al*., 2019).

In all applications, it remains the duty of a sensory professional to draw conclusions from the elucidation of information generated by the statistical analysis of the raw data (de Pelsmaker *et al*., 2019).

12. CONCLUSION

With novelty being a driving factor in the ever-expanding food and beverage industry, researchers have turned to South African botanicals as a source of potentially valuable flavours, offering potential functionality and nutritional advantages (Gruenwald, 2009; Van Wyk, 2011).

Many of these South African botanicals have a long history of use in traditional applications, particularly for their medicinal properties (Joubert *et al*., 2011). A few indigenous South African plants have reached enormous commercial success, with rooibos tea and

Amarula® cream liqueur being two very well-known examples (Le Maitre *et al*., 1997; Van Wyk, 2011).

Increased consumer awareness to the link between nutrition and welfare and longevity has led to the movement away from synthetic ingredients toward more natural alternatives, thereby creating a gap in the market for extracts from traditional African botanicals (Gruenwald, 2009). The full potential of many botanicals has not been realised, for example, in the case of Baobab (*Adansonia digitata*), Moringa (*Moringa oleifera*), Honeybush (*Cyclopia genistoides*) and Pepperbark (*Warburgia salutaris*) (Gruenwald, 2009; Kamatou *et al.,* 2011; Oyeyinka & Oyeyinka, 2015; Joubert *et al*., 2017).

All four botanicals of interest have been reported as safe for consumption and have been extensively researched for their pharmacological effects, however, limited information about their sensory properties is available, except for *Cyclopia* spp., for which a sensory wheel has been established (Osman, 2004; Joubert *et al.*, 2008; Chadare *et al*., 2009; PhytoTrade Africa, 2009; Kamatou, Vermaak & Viljoen, 2011; Theron *et al*., 2014; Leone *et al.*, 2015; Stohs & Hartman, 2015; Joubert *et al.*, 2017; Matic *et al*., 2018; Tiloke *et al.,* 2018).

Exploration of the tastes and flavours of plant extracts is made possible by the novel separation technique, LC Taste® (Reichelt *et al*., 2010a; Reichelt *et al*., 2010b; Reichelt *et al*., 2010c). Through fine-tuning the multiple parameters that affect HTLC separation speed and efficiency, a rapid screening method can be developed to identify taste-active fractions without prior isolation of compounds (Reichelt *et al*., 2010a; Reichelt *et al*., 2010b; Reichelt *et al*., 2010c).

Coupled to sensory analysis, different aspects of the individual fractions can be assessed to gain valuable consumer and potential product information (Civille & Oftedal, 2012; Yu *et al.*, 2018). In this way, the protocol can be applied to the herbal extracts to uncover fractions with appealing flavours and tastes or to remove fractions with negative sensory attributes, thereby improving the flavour of the combined remaining fractions (Reichelt *et al.*, 2010a; Mittermeier *et al.,* 2018).

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CHAPTER 3
LC TASTE® METHOD DEVELOPMENT FOR THE PRELIMINARY SCREENING OF BOTANICALS FOR POTENTIALLY VALUABLE FLAVOURS

ABSTRACT

This research chapter applies the separation technique, LC (Liquid Chromatography) Taste®, as rapid screening method to identify potentially valuable flavour fractions from a range of South African botanicals, specifically, *Adansonia digitata* (Baobab), *Cyclopia intermedia* (Honeybush), *Moringa oleifera* (Moringa) and *Warburgia salutaris* (Pepperbark). Concentrated extracts of the preferred flavour from each botanical were prepared using several exploratory steps. The concentration of ethanol (0%, 50%, 100%, $v.v^{-1}$) in the aqueous solvent, the solid-liquid ratio (15%, 25%, 50%, $w.v^{-1}$), the effect of sonication versus maceration, and the effect of the duration of the extraction time (3 h, 6 h, 24 h) at elevated temperature (70°C) on extraction efficiency was explored. The decision of flavour extraction efficiency was made based on sensory evaluation as well as the relevant peak intensities of the extracts on high performance liquid chromatography-diode-array detection (HPLC-DAD) chromatograms. It was found that 50% ethanol concentration ($v.v^{-1}$) extracted the preferred flavour from each botanical, confirming that hydroalcoholic solvents of water and ethanol are highly effective in their extraction of phytochemicals from a range of plant-based matrices. The maximum solid-liquid ratio for Moringa was 15% ($w.v^{-1}$), while 25% could be used for Baobab, Pepperbark and Honeybush. Although the maximum solid-liquid ratio produced concentrated extracts, the concentration of solids should preferably be optimised per botanical to prevent solvent saturation. Concerning the extraction techniques, maceration with magnetic stirring led to better extraction of flavour from Honeybush, Moringa and Baobab, while sonication resulted in better flavour extraction in the case of Pepperbark. Extraction temperature should enhance extraction efficiency but should not have a detrimental effect on the plant material. It was found that 24 h was the best for flavour extraction from Honeybush, suggesting that the compounds responsible for flavour in Honeybush tea are not highly sensitive to thermal degradation. In contrast, the taste- and peak intensities of Pepperbark, Baobab, and Moringa, decreased with increasing extraction time at elevated temperature (70°C), suggesting that the compounds responsible for the taste of these botanical samples are more susceptible to thermal degradation. The preferred extract from each botanical was prepared and separated via LC Taste® fractionation, using a Hamilton Polymeric column. The high-performance liquid chromatography (HPLC) instrument was equipped with a DAD detector and a Polaratherm column heater set to 80°C. The mobile phase gradient consisting of (A) 0.1% formic acid in water ($v.v^{-1}$) and (B) ethanol, adjusted per botanical. One-minute fractions were collected based on the established fraction collection window, selected based on the occurrence of DAD

signals as a function of time. The fractions were collected for the tasting panel to screen the various fractions eluted from each botanical for potentially valuable flavours. LC Taste® proved to be a useful, rapid method for screening the flavours and tastes present in each of the botanicals, without dominant bitterness or sourness from adjacent fractions overpowering the flavours unique to each fraction. Although the chromatograms were relatively poorly resolved, the aim of the LC Taste® experiments - to rapidly screen a complex extract for interesting flavours and/or tastes - was achieved. Further investigation of fractions is required to identify the specific compounds responsible for the perceived sensory results. To ensure microbial safety of the fractions before tasting, they were tested for the presence of *Salmonella* spp. based on the International Organization for Standardization (ISO) 6579: - 1:2017 method, as well as for *Bacillus cereus* spores using an adapted version of the ISO 7932:2004 method. The microbial results of this study indicated that the botanical fractions were all free from the pathogenic microbes, suggesting that the obtained fractions are microbiologically safe for human tasting.

1. INTRODUCTION

Botanical products and preparations have become increasingly popular ingredients in the international food and beverage industry in recent years as consumer awareness and science-based evidence of their inherent health properties has grown (Harnly *et al.,* 2017; Mantzourani *et al.*, 2019; Nazir *et al*., 2019). Many undiscovered flavours from the wide array of southern African botanicals have the potential to be used as ingredients in food and beverages products (Van Wyk, 2011).

In order to evaluate ingredients for potentially valuable flavours, the flavour industry requires methods to screen different substrates, preferably in a manner that is not excessively laborious or time-consuming.

The conventional approach to screen non-volatile components in a complex natural matrix, such as a botanical extract, would require the fractionation and isolation of the compounds via liquid chromatography, such as preparative HPLC or liquid-liquid partition chromatography, and would typically require the use of toxic solvents (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c).

Fractions isolated from conventional HPLC analyses can therefore not be directly used for human taste evaluations and thus time-consuming and often expensive procedures are necessary before the isolated compounds can be safely tasted by a tasting panel (Reichelt *et al*., 2010b; Yabré *et al*., 2018).

In order to provide a more rapid and efficient alternative to overcome the limitations of conventional separations of food mixtures, Symrise (2005) developed the LC Taste® methodology. LC Taste® is a flavour screening method that enables the analytical separation of non-volatile compounds in complex food matrices via reverse phase-high temperature liquid chromatography (RP-HTLC). The process allow for simultaneous or direct sensory evaluation of eluted fractions (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c).

LC Taste® utilises water as the primary mobile phase component, although other non-toxic compounds, for example, ethanol, may be incorporated (Reichelt *et al*., 2010). Elevated temperatures are often used to reduce the viscosity of ethanolic mobile phases, thereby avoiding pressure constraints (Reichelt *et al*., 2010).

The relation of analytical to sensory data is made possible without the need of subsequent steps to remove harmful solvents, thereby protecting chemical constituents from deterioration and chemical changes (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c).

Correlation of the sensory data with analytical detection, for example, liquid chromatography–mass spectrometry (LC-MS) or liquid chromatography–diode-array detection (LC-DAD), allows structural elucidation and quantification, if required (Reichelt *et al.,* 2010b; Mittermeier *et al.,* 2018).

LC Taste® is therefore a useful technique to rapidly screen solutions for interesting flavour fractions, without dominating flavours or tastes from adjacent fractions interfering with the flavour or taste unique to a specific fraction (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c). Once fractions of interest are identified, further analytical investigation can be used to identify the compound(s) responsible for the flavour detected (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c).

Before LC Taste® can be applied to effectively identify flavour- or taste-active fractions from the botanicals, the extracted flavour from each botanical must be intensified. For successful extraction of secondary metabolites from botanical ingredients, the plant part, its physicochemical properties and tissue matrix type are all factors that need to be considered (Azmir *et al*., 2013; Belwal *et al*., 2018). The cell structure, form of target compounds, moisture content, and particle size are some of the most important properties of a plant to be extracted (Pronyk & Mazza, 2009; Belwal *et al*., 2018; Anbalagan *et al.*, 2019; Zhang *et al.*, 2019).

Critical extraction parameters that affect the nature and yield of the extracted analytes include the choice of solvents applied, the extraction temperature and duration and the solid-liquid ratio (Anbalagan *et al.*, 2019; Dirar *et al.*, 2019; Zhang *et al*., 2019). The extraction temperature and duration should strike a balance between the extraction efficiency and thermal stability of the active compounds (Chan, Yusoff & Ngoh, 2014; Nastic *et al.,* 2018).

Conventional techniques to extract bioactive compounds from botanical sources are generally based on the extracting power of different solvents and the application of heat and/or mixing (Azmir *et al*., 2013). In order to obtain bioactive compounds from plants, the existing classical techniques are Soxhlet extraction, maceration and hydrodistillation (Azmir *et al*., 2013).

With the growing demand for herbal products and extracts for wider and safer applications, the challenge is to provide readily available, high-quality products with low cost of processing and higher yield (Belwal *et al*., 2018). For this reason, there is an increased demand for alternative extraction techniques (Belwal *et al*., 2018). Alternative techniques used for this purpose include microwave assisted extraction (MAE), supercritical fluid extraction (SFE), pressurised liquid extraction (PLE), ultrasonic assisted extraction (UAE), pulsed electric field assisted extraction (PEF), enzyme assisted extraction (EAE), among others (Belwal *et al*., 2018). These techniques are known for their reduced extraction time and volume of organic and toxic solvents, their simplicity and enhanced extraction yields with lower energy consumption, making them more environmentally friendly (Belwal *et al*., 2018).

As with any ingredient intentionally added to food, microbial safety is an important parameter to consider in botanical extracts (Trucksess & Scott, 2008; Thanh *et al*., 2018). Since raw materials for botanical preparations are typically traded in dried form, they are associated mainly with bacterial endospores and fungal spores, capable of surviving low

humidity conditions (Warude & Patwardhan, 2004; EHIA, 2008; Fogele *et al*., 2018; Székács *et al.,* 2018; Thanh *et al*., 2018). From the broad-spectrum of microbes and fungi that have been associated with herbal products, pathogens raise the most concern due to their potential detrimental impact on human health (Warude & Patwardhan, 2004; Székács *et al.,* 2018).

Well known pathogenic microbes associated with botanical products are *Escherichia coli, Listeria monocytogenes, Salmonella typhi, Pseudomonas aeruginosa* and *Staphylococcus aureus* (Warude & Patwardhan, 2004; Harnly *et al*., 2017; Chugh *et al.*, 2018). Microorganisms associated with herbal products that are most resistant to drying are the spore-forming *Bacillus cereus, Clostridium perfringens, Clostridium botulinum*, and moulds, such as *Aspergillus spp.,* and *Penicillium* spp*.,* which are potential producers of a variety of toxins (Witkowska *et al*., 2011; Ainiza *et al.,* 2015; Fogele *et al.,* 2018; Thanh *et al*., 2018).

B. cereus is an extremely tenacious microorganism, surviving various stress conditions, including thermal stress and sanitary practice, through its ability to form endospores (Ceuppens *et al.*, 2011; Fogele *et al*., 2018). *B. cereus* is a toxin-producing human pathogen that results in diarrheal and emetic forms of food poisoning (Ceuppens *et al.*, 2011; Fogele *et al*., 2018). Diarrheal food poisoning occurs after consumption of higher cell or spore dosages, typically greater than 10^5 (colony-forming units per gram) $CFU.g^{-1}$, resulting in enterotoxin production in the gut (Thanh *et al*., 2018).

Microbial contamination can be introduced either during, pre- or post-harvesting (Ndhlala *et al.,* 2018). The risk is of contamination is greater when there is a lack of quality control and monitoring systems during the production of herbal products (Chugh *et al*., 2018). Plants that have been grown in polluted environments or farmed negligently, could potentially be unsafe for human consumption (Chugh *et al*., 2018). Similarly, inappropriate storage conditions negatively affect the quality of herbal products, especially if they are subjected to fungal or bacterial contamination (Chugh *et al*., 2018). It has been found that warm, humid conditions encourage the growth of moulds and the production of toxins such as mycotoxins (Trucksess & Scott, 2008). Mould growth can therefore be prevented by implementing strict hygiene and operational guidelines during the harvesting and storage of botanicals (Trucksess & Scott, 2008).

The microbial risks associated with botanical preparations varies depending on the production stage since processing factors contribute to the microbial load of the resulting product (Warude & Patwardhan, 2004; Dao & Dantigny, 2011; Székács *et al.,* 2018). Hot water or ethanol extractions significantly reduces the viable counts by multiple log units and deactivates possible pathogens and toxins (Warude & Patwardhan, 2004; EHIA, 2008; Dao & Dantigny, 2011; Chugh *et al*., 2018).

Considering the opportunities available for botanical flavours, the research aim of this chapter was to identify one or more potentially valuable flavours from a range of southern

African botanicals, namely Baobab (*Adansonia digitata*), Moringa (*Moringa oleifera*), Honeybush (*Cyclopia genistoides*) and Pepperbark (*Warburgia salutaris*) by applying the novel flavour screening technique, LC Taste®. Objectives of this research chapter were to manipulate the extraction conditions for each of the botanicals to produce concentrated extracts of the preferred flavour profile from each and to optimise the LC Taste® protocol for each botanical. The microbial safety of the collected flavour fractions from each botanical sample were to be proven before sensory evaluation as an additional objective of this chapter.

In order to achieve the aim and objectives set out for this chapter, LC Taste® will be applied to the mentioned botanicals to explore their flavour potential. Exploratory steps will be applied to each of the botanicals to intensify the flavour extracted from each. The concentration of ethanol in the aqueous solvent, the solid-liquid ratio, the effect of sonication versus maceration, and the effect of the duration of the extraction time at elevated temperature on extraction efficiency will be evaluated. The decision of flavour extraction efficiency will be made based on sensory evaluation in addition to the peak intensity of the extracts on generated diode-array detection (DAD) chromatograms. The eluted flavour fractions will be subjected to *Bacillus cereus* and *Salmonella* microbial tests to ensure their microbial safety before tasting.

2. MATERIALS AND METHODS

2.1 Botanical samples

Samples of *Adansonia digitata* (Baobab) dried fruit pulp, *Moringa oleifera* (Moringa) dried leaf powder and *Warburgia salutaris* (Pepperbark) dried leaf powder were obtained from Kerry Foods (Hillcrest, Durban, South Africa). A commercially prepared, fermented sample of *Cyclopia genistoides* (Honeybush) loose tea leaves was obtained from ARC Infruitec (Stellenbosch, South Africa). The samples were stored in sealed containers in dry conditions, at ambient temperature, out of direct sunlight.

2.2 Experimental design

Several exploratory steps were required to determine the solvent composition, solids concentration and extraction technique and time that produces the most concentrated extract of the preferred flavour profile from each botanical sample. The effect of the concentration of ethanol in the aqueous solvent on the extraction of specific flavours from each botanical was first explored. Next, the percentage of solids was increased to a maximum level for each botanical in the selected solvent, provided that fluid could still be filtered from the botanical-solvent mixtures after sonication. Finally, sonication was compared to maceration with stirring based on the intensity of the flavour extracted from each botanical, as was the effect of stirring the extracts for different time periods at a constant temperature.

Preliminary analytical separations were performed for each of the botanicals, generating chromatograms of peak intensity as a function of analysis time to provide an idea of the elution pattern and the peak intensities of the different samples before they were subjected to the LC Taste® fractionation.

The preferred extract from each botanical was prepared and fractionated by LC Taste®, using a polymeric column. Once the chromatographic conditions were established for the LC Taste® separation of each of the botanicals, the fraction collection window for each was selected based on their DAD chromatograms. Fractions were collected from each botanical for a preliminary tasting panel to screen for potentially valuable flavours. The preliminary tasting was done to determine whether distinguishable flavours could be detected by untrained individuals.

To ensure microbial safety, the LC Taste® fraction of each botanical with the lowest concentration of ethanol, considered as the least safe fraction to be subjected to sensory analysis, was collected for microbial testing.

2.3 Extraction conditions

Three extracts were made from each botanical by extracting 1 g with 20 mL of ethanol (≥99.9%, LiChrosolv, Sigma-Aldrich Merck, Darmstadt, Germany) and deisonised water in three different ratios, namely pure ethanol, 1:1 ethanol:water ($v.v^{-1}$) and pure deionised water. Each of the samples were sonicated at 40°C for 90 minutes (Hwashin Ultrasonic Cleaner, Power Sonic410, frequency 40 kHz, capacity 10 L, required power 400 W, Seoul, Korea) in 100 mL Schott bottles, after which the samples were filtered through filter paper (MN 615, 0.16 mm, weight 70 $g.m^{-2}$, average filtration capacity 4-12 µm, ash content 0.1%, Macherey-Nagel & Co., Germany) with a vacuum pump (0.12 kW, 50/60 Hz, 230 + 10% V; ABM Greiffenberger Antriebstechnik GmbH, Marktredwitz, Germany) to remove the solids from the extract. The remaining extract was transferred to a round-bottom flask and the ethanol was removed from each sample on a rotary evaporator at 40°C, after which samples were diluted to 20 mL for tasting using bottled water.

To maximise the solids concentration of the extraction, different amounts of each botanical (3 g, 5 g, and 10 g) were extracted with 20 mL of a 50% ethanol solution ($v.v^{-1}$), yielding solids concentrations of 15%, 25% and 50% ($w.v^{-1}$), respectively. The sonication temperature and time was increased to 70°C for 3 h.

Continuing with the maximum solids concentration of each botanical, the samples were subjected to different extraction techniques and times to enhance flavour extraction. One sample of each botanical was sonicated at 70°C for 3 h while one was stirred using a magnetic stirrer (Velp® Scientifica GDE Heated Circulating Bath, setting 8, Vanderbijlpark, South Africa) for 3 h in a water bath set at 70°C. Another two samples of each botanical were stirred at 70°C

for 6 h and 24 h, respectively. Extractions were performed in 100 mL Schott bottles with tightly screwed lids. The samples were filtered into a round-bottom flask using a vacuum pump and filter paper and the ethanol was subsequently removed from the samples on a rotary evaporator at 40°C. The remaining extract of each botanical was reconstituted with deionised water to a defined volume before being further filtered through a 0.45 µm hydrophilic PVDF (polyvinylidene difluoride) syringe membrane filter (0.45 µm, 33 mm, Agela Technologies, Torrance, California) into 1.5 mL sample vials for HPLC analysis.

The preferred extraction technique was selected for each botanical based on chromatograms of the samples analysed on a Luna C_{18} column (150 × 4.6 mm, 5 µm) (Phenomenex, Torrance, California, U.S.) (refer to section 2.4 for details) by considering the relative peak intensities as well as by comparing the taste intensities of each of the samples.

2.4 Analytical chromatographic separations

Before sample fractionation, analytical separations were performed for each of the botanicals on a Luna C_{18} column (150 × 4.6 mm, 5 µm) at room temperature, with a flow rate of 1 $mL.min^{-1}$. The mobile phase consisted of (A) 0.1% formic acid in water ($v.v^{-1}$) and (B) acetonitrile (Gradient-grade, Merck, Darmstadt, Germany). A gradient of 0% to 100% B was set in a determined time interval. An initial injection volume of 10 µL was employed and the solvent gradient was adjusted per botanical. The experiment was performed on an HP 1050 Series HPLC instrument (Hewlett-Packard, Palo Alto California, U.S.), consisting of a pump, autosampler and DAD detector and an Agilent 1100 Series degasser (Agilent Technologies, Waldbronn, Germany). Chromatograms of peak intensity as a function of time were collected at 254 nm and 280 nm. Data was collected via ChemStation software (Agilent Technologies, Waldbronn, Germany).

2.5 LC Taste® fractionation

For LC Taste® separation, separations for each botanical sample were performed on a Hamilton Polymeric (PRP-1) column (250 × 10 mm, 10 µm) (Hamilton company, Nevada, U.S.) with an injection volume of 1 000 µL and a solvent flow rate of 3 $mL.min^{-1}$. The mobile phase consisted of (A) 0.1% formic acid in water ($v.v^{-1}$) and (B) ethanol. The experiments were performed on an HP 1050 Series HPLC instrument, containing a pump, autosampler and DAD detector, set to collect signals at 254 and 280 nm. The system was equipped with an Agilent 1100 Series degasser and a Polaratherm column heater (Polaratherm, Series 9000, Sandra Selerity Technologies Inc., Salt Lake City Utah, U.S.) set to 80°C. Data was collected via ChemStation software and one-minute fractions were collected with a fraction collector (Model 2110, Bio-Rad, Hercules, California, U.S.).

2.5.1 *Adansonia digitata*

The extract to be injected was prepared by magnetically stirring the 25% solids concentration (w.v^{-1}; 5 g.20 mL^{-1}), with a 50% (v.v^{-1}) aqueous ethanol solvent (20 mL), at 70°C for 3 h. The extract was filtered into a round-bottom flask using a vacuum pump and filter paper and the ethanol was subsequently removed from the sample on a rotary evaporator at 40°C. The remaining extract was reconstituted with deionised water to a volume of 2.5 mL before being further filtered through a 0.45 µm hydrophilic PVDF syringe membrane filter into 1.5 mL sample vials for HPLC analysis.

The mobile phase consisted of (A) 0.1% formic acid in water (v.v^{-1}) and (B) ethanol, with a linear gradient from 0% to 100% B in 25 min and one-minute fractions were collected between 2 and 17 minutes with a fraction collector.

2.5.2 *Cyclopia genistoides*

The Honeybush extract for LC Taste® injection was prepared by magnetically stirring the 25% solids concentration (w.v^{-1}; 5 g.20 mL^{-1}), with a 50% (v.v^{-1}) aqueous ethanol solvent (20 mL), at 70°C for 24 h. The extract was filtered, and the ethanol evaporated as described for *Adansonia digitata* (refer to 2.5.1). The remaining extract was reconstituted with deionised water to a volume of 4 mL before being further filtered into sample vials for HPLC analysis (as described in 2.5.1).

The mobile phase consisted of (A) 0.1% formic acid in water (v.v^{-1}) and (B) ethanol, with a linear gradient from 0% to 100% B in 25 min and one-minute fractions were collected between 4 and 19 minutes.

2.5.3 *Moringa oleifera*

The injected Moringa extract was prepared by magnetically stirring the 15% solids concentration (3 g.20 mL^{-1}), with a 50% (v.v^{-1}) aqueous ethanol solvent (20 mL), at 70°C for 3 h. The extract was filtered and the ethanol evaporated at 40°C (refer to 2.5.1). The remaining extract was reconstituted with deionised water to a volume of 4 mL before being further filtered into sample vials for HPLC analysis (as described in 2.5.1).

The mobile phase consisted of (A) 0.1% formic acid in water (v.v^{-1}) and (B) ethanol, with a linear gradient from 0% to 70% B in 20 min, and 70% B to 100% B in an additional 5 minutes (20–25 min). One-minute fractions were collected between 3 and 18 minutes.

2.5.4 *Warburgia salutaris*

The injected extract was prepared by sonicating the 25% solids concentration (w.v^{-1};5 g.20 mL^{-1}), with a 50% (v.v^{-1}) aqueous ethanol solvent (20 mL), at 70°C for 3 h. The extract was filtered, and the ethanol evaporated on a rotary evaporator at 30°C. The remaining extract

was reconstituted with deionised water to a volume of 6.5 mL before being further filtered into sample vials for HPLC analysis (as in 2.5.1).

The mobile phase consists of (A) 0.1% formic acid in water ($v.v^{-1}$) and (B) ethanol, with a linear gradient from 0% to 100% B in 25 min and one-minute fractions were collected between 6 and 17 minutes.

2.6 Preliminary tasting

For the preliminary tasting of extracts and fractions, samples were initially tested by a group of 6 untrained individuals (3 males, 3 females), all with previous experience in sensory evaluation of food and beverage products.

For each sensory evaluation, the participants were presented with bottled water and unsalted crackers to cleanse their pallets between tastings. The samples were presented to the participants in polystyrene cups in randomised order.

For the determination of the suitable solvent composition for each botanical, 3 mL of each extract (100% ethanol; 50% ethanol and 100% deionised water; $v.v^{-1}$) from each botanical was presented to the participants. As previously mentioned (refer to 2.3), the ethanol had been removed (40°C) and the extracts were rediluted to 20 mL using bottled water at room temperature. The participants were asked to indicate the preferred extract from each botanical.

To determine which extraction technique was the most suitable for each botanical, 3 mL of each of the sonicated and the magnetically stirred extract (50% ethanol; $v.v^{-1}$) from each botanical was presented to the 6 participants. The ethanol had been removed (40°C) from these extracts and they were rediluted to 20 mL using bottled water at room temperature. The participants were asked to indicate the more intense extract between the two.

Similarly, three extracts from each botanical, stirred for 3 h, 6 h and 24 h respectively, were evaluated for flavour intensity. After stirring the extracts at 70°C for the stipulated times, the ethanol was removed using rotary evaporation at 40°C and the extracts were reconstituted to 20 mL using bottled water at room temperature. A 3 mL volume of each extract was presented to the panel and they were asked to rank the three samples from each botanical in order of increasing flavour intensity.

For the preliminary flavour screening of the fractions collected from each botanical by LC Taste®, the same individuals were presented with 1.5 mL of each collected ethanol-containing fraction in randomised order. The participants were asked to indicate whether they detected a significant taste from the fraction (other than ethanol), and to describe the taste where possible.

The ethanol-containing fractions were sent to Durban where they were tasted by an expert sensory panel from Kerry Foods consisting of 5 people, all with extensive knowledge and training in flavours.

2.7 Microbiological testing of the botanical fractions

2.7.1 Sample acquisition, preparation and storage

Fraction 2 of Baobab, fraction 4 of Honeybush, fraction 3 of Moringa and fraction 6 of Pepperbark were collected as described in the LC Taste® fractionation section (2.5) above. Three repetitions of each fraction were collected, and the ethanol was subsequently removed from the combined fractions of each botanical using rotary evaporation at 40°C under vacuum.

The aqueous extracts of the four botanicals were transferred to sample vials and (at 4°C) before microbial analysis the following day (24 h later).

2.7.2 Detection of *Salmonella* spp. and *Bacillus cereus*

The botanical fractions were tested for the presence of *Salmonella* spp. based on the International Organization for Standardization (ISO) 6579: - 1:2017 method. The four fractions, one from each botanical, were placed in buffered peptone water (1:10 $v.v^{-1}$) (Oxoid, South Africa), mixed via centrifugation and then incubated at 37°C for 16-20 h. After incubation, 0.1 mL of the buffered peptone water pre-enrichment was transferred to 10 mL Rappaport Vassiliadis Soya (RVS) broth (Oxoid, South Africa) in screw top Schott bottles. The bottles containing the enrichment broth were incubated for 18-24 h at 41-43°C. Using the culture obtained from the enrichment broth after incubation, an inoculation loop was used to streak the surface of pre-dried Xylose Lysine Deoxycholate (XLD) agar plates (Oxoid, South Africa), in duplicate. The plates were inverted and incubated for 24 h at 35°C before examining the plates for typical colonies of *Salmonella*. The plates were incubated under the same conditions for an additional 18-24 h before examining them for a second time.

The botanical fractions were tested for the presence of *Bacillus cereus* spores using an adapted version of the ISO 7932:2005 method. Using aseptic pipetting, each of the four fractions was diluted with buffered peptone water in a 1:10 ($v.v^{-1}$) ratio and vortexed for 2 minutes. A 10^{-2} dilution was prepared for each botanical by transferring 10 mL homogenised sample to 90 mL buffered peptone water. The 10^{-1} and 10^{-2} dilutions were placed in a water bath at 70°C for 15 minutes. Duplicate Mannitol-egg yolk-polymyxin (MYP) (Oxoid, South Africa) agar plates were prepared for both dilutions of each fraction by spreading 0.1 mL evenly onto the surface of each plate with a sterile glass spreading rod. The plates were incubated at 30°C for 18-24 h. After examination, the plates were incubated under the same conditions for another 24 h before examining the plates for growth for a second time.

3. RESULTS

3.1 Extraction conditions

3.1.1 *Adansonia digitata*

The entire untrained sensory panel (100%) preferred the flavour extracted from *Adansonia digitata* by the 50% aqueous ethanol ($v.v^{-1}$) solvent, compared to the 100% ethanol and pure deoinised water extracts.

The highest solids concentration of *Adansonia digitata* from which a fluid extract could be obtained for further analysis was at 25% ($w.v^{-1}$). At 50% solids concentration ($w.v^{-1}$), no extract could be retrieved from filtering the botanical-solvent mixture after sonication at 70°C for 3 h since the resulting product was a dry, solid mass.

The analytical chromatogram of the 25% solids ($w.v^{-1}$) sample, magnetically stirred in a water bath at the same temperature (70°C), for the same time-period (3 h) as sonication, yielded more intense peaks than the sonicated sample (Figure 3.1).

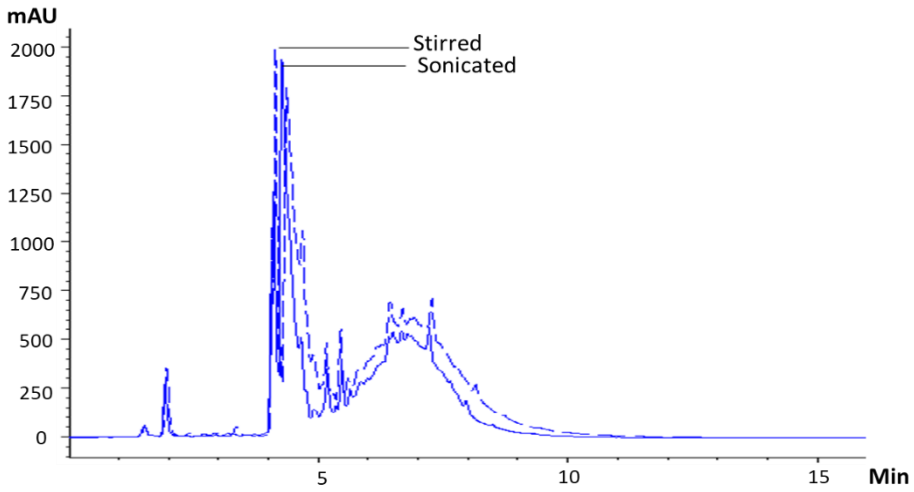

Figure 3.1 Chromatograms (254 nm) comparing the peak intensities of *Adansonia digitata* samples stirred or sonicated at the same temperature (70°C) for the same time period (3 h)

The untrained sensory panel could not taste a big difference in the taste intensities of the two samples (sonicated compared to stirred), however more of the participants (67%) indicated that a stronger flavour was detected in the stirred sample, agreeing with the peak intensities visible on the chromatographic representation (Figure 3.1).

Comparing the peak intensities of the different stirring times in the resulting chromatogram in Figure 3.2, below, the most intense peaks alternate between the samples stirred for 3 hours and 6 hours. The *Adansonia digitata* sample stirred for 24 hours, however, consistently resulted in the least intense peaks (Figure 3.2).

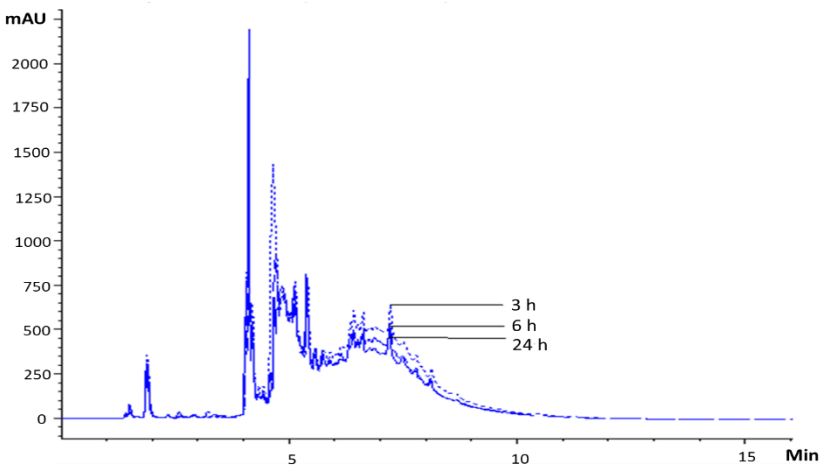

Figure 3.2 Chromatograms (254 nm) comparing the peak intensities of *Adansonia digitata* samples stirred in a water bath at 70°C for different times

Informal tasting by the untrained sensory panel indicated that for 83% of the sensory panel, the sample stirred for 3 h was the most intense in terms of taste, followed by the samples stirred for 6 h and 24 h, respectively. The taste results reflected the results represented by the chromatogram (Figure 3.2).

3.1.2 *Cyclopia genistoides*

The majority of the untrained sensory panel (83%) indicated that 50% aqueous ethanol ($v.v^{-1}$) extracted the preferred flavour from *Cyclopia genistoides.*

The highest solids concentration of *Cyclopia genistoides* from which a fluid extract could be obtained for further analysis was at 25% ($w.v^{-1}$). After sonicating a 50% solids concentration ($w.v^{-1}$) of Honeybush, no liquid could be filtered from the extract.

The chromatograms of the 25% solids ($w.v^{-1}$) samples, comparing the peak intensities of a sonicated and magnetically stirred sample extracted at the same temperature (70°C), for the same time-period (3 h), resulted in fairly similar chromatograms for *Cyclopia genistoides* (Figure 3.3).

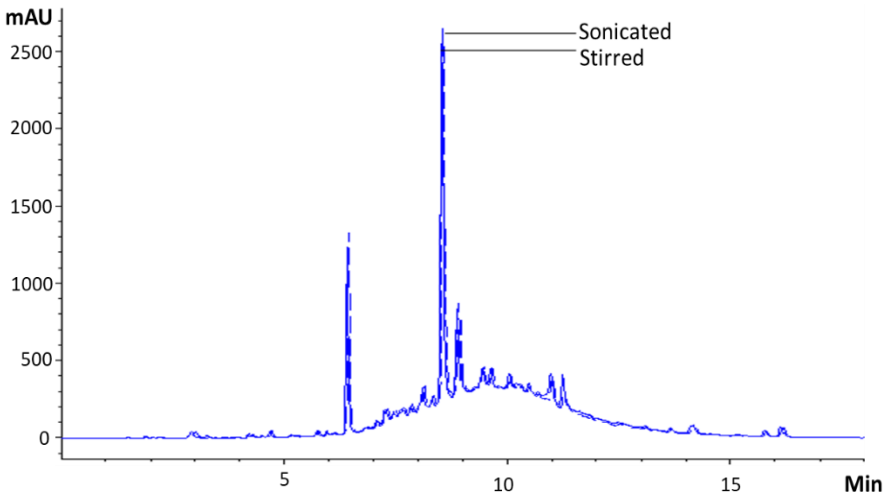

Figure 3.3 Chromatograms (280 nm) comparing the peak intensities of *Cyclopia genistoides* samples stirred or sonicated at the same temperature (70°C) for the same time period (3 h)

The taste intensities of the *Cyclopia genistoides* samples stirred and sonicated for the same time and at the same temperature were indicated as similar by the untrained taste panel, who could not detect a difference in the flavour intensity between the samples, correlating with the results of peak intensity in Figure 3.3.

Comparing the peak intensities of the different stirring times in the resulting chromatogram in Figure 3.4, below, it can be seen by the large degree of overlap that there is a very small difference in the peak intensities between the three trials. Some peaks are more intense for the 3 h and 6 h stirring times, whereas most of the peaks eluting after 9 minutes are more intense in the sample stirred for 24 h (Figure 3.4).

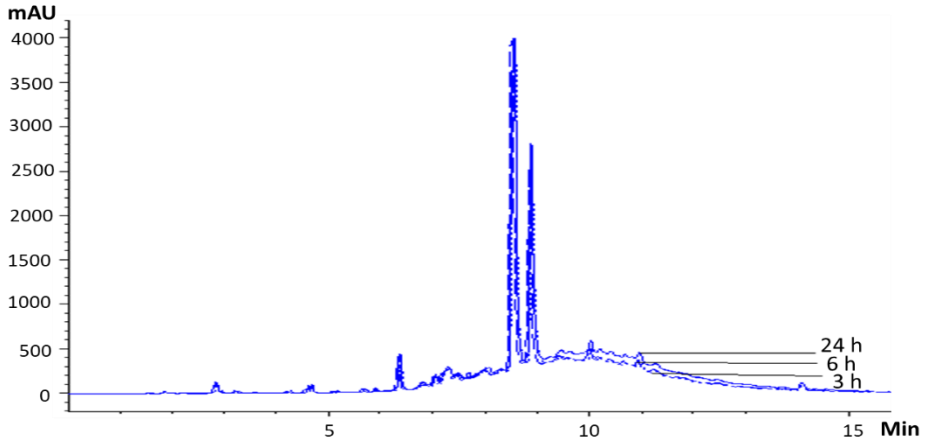

Figure 3.4 Chromatograms (280 nm) comparing the peak intensities of *Cyclopia genistoides* samples stirred in a water bath at 70°C for different times

The untrained sensory panel could not detect a difference in the taste intensity of the samples of Honeybush processed for different times, as indicated by the large degree of overlap of the chromatographic peaks (Figure 3.4).

3.1.3 *Moringa oleifera*

The greater part of the untrained sensory panel (83%) indicated that 50% aqueous ethanol ($v.v^{-1}$) extracted the preferred flavour from *Moringa oleifera.*

Above the solids concentration of 15% ($w.v^{-1}$), no fluid could be extracted from the botanical-solvent mixture after sonication at 70°C for 3 h. Therefore, 15% ($w.v^{-1}$) was selected as the highest solid-liquid ratio for Moringa.

The analytical chromatograms of the 15% solids ($w.v^{-1}$) sample, either sonicated or magnetically stirred, yielded relatively similar chromatograms for *Moringa oleifera*, with the most intense peaks alternating between the sonicated and stirred sample (Figure 3.5).

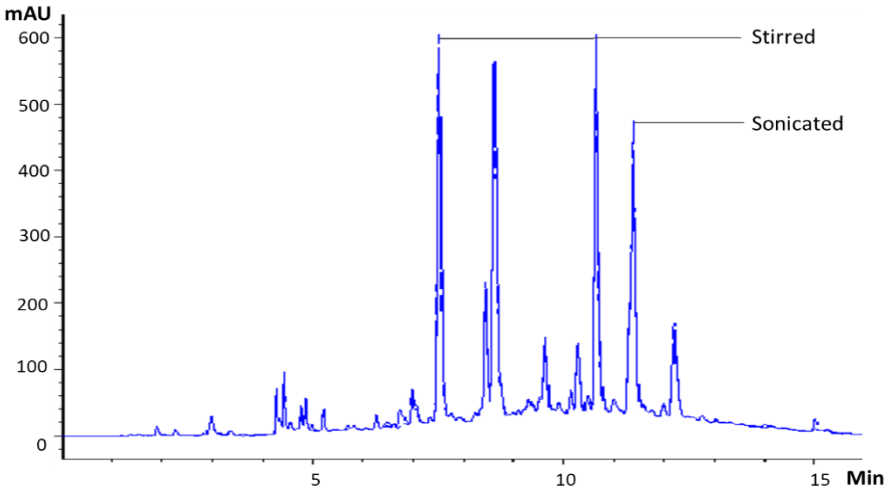

Figure 3.5 Chromatograms (280 nm) comparing the peak intensities of *Moringa oleifera* samples stirred or sonicated at the same temperature (70°C) for the same time period (3 h)

Tasting by the untrained sensory panel showed that taste intensities of the sonicated sample and the stirred sample were similar, which was the expected result based on Figure 3.5.

Comparing the peak intensities of the three different stirring times in the resulting chromatogram in Figure 3.6, below, it can be seen that the sample stirred for 3 hours yielded the most intense peaks, followed by 6 hours, and lastly, the 24-hour stirring time.

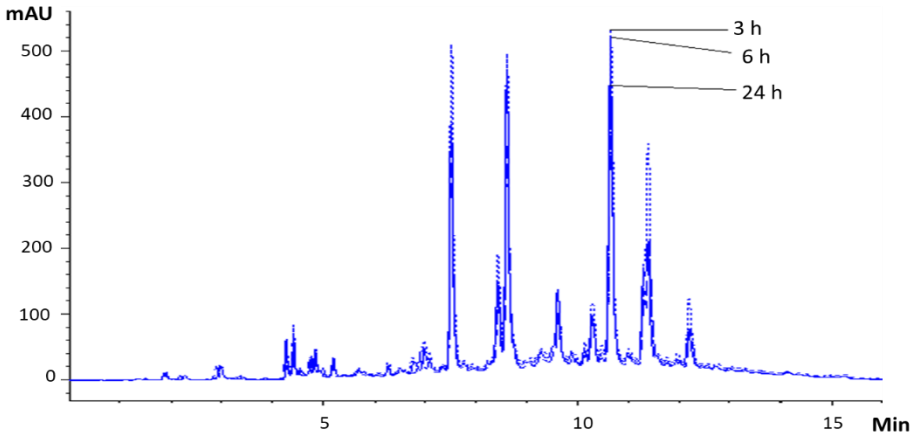

Figure 3.6 Chromatograms (280 nm) comparing the peak intensities of *Moringa oleifera* samples stirred in a water bath at 70°C for different times

The taste intensity of the sample stirred for 3 h was indicated by 67% of the untrained panel as the most intense of the three samples, reflecting the result of the chromatographic peak intensity (Figure 3.6).

3.1.4 *Warburgia salutaris*

The full untrained sensory panel (100%) indicated that 50% aqueous ethanol ($v.v^{-1}$) extracted the preferred flavour from *Warburgia salutaris.*

The highest solids concentration of *Warburgia salutaris* from which a fluid extract could be obtained for further analysis was at 25% solids concentration ($w.v^{-1}$). After sonicating the 50% solids concentration ($w.v^{-1}$) botanical sample, a solid mass resulted from which no fluid could be retrieved.

The analytical chromatograms of the 25% solids ($w.v^{-1}$) sample resulted in less intense peaks for the stirred sample than the *Warburgia salutaris* sample that was sonicated (Figure 3.7).

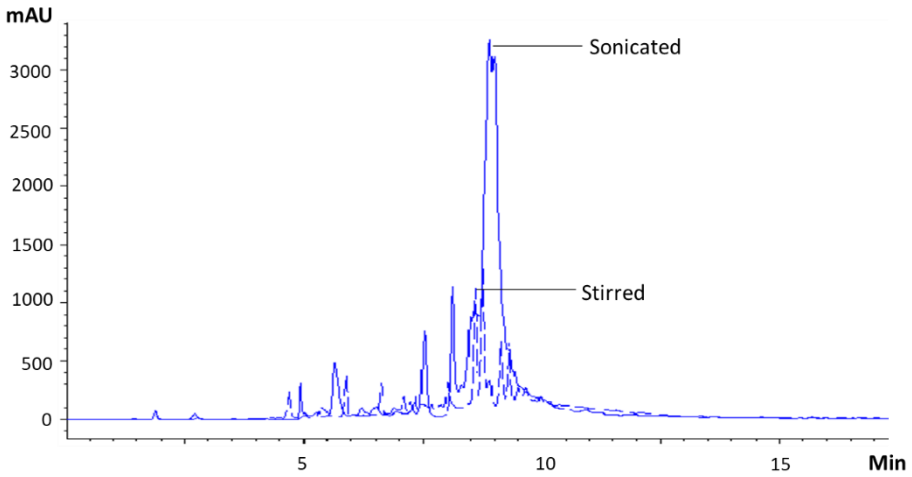

Figure 3.7 Chromatograms (254 nm) comparing the peak intensities of *Warburgia salutaris* samples stirred or sonicated at the same temperature (70°C) for the same time period (3 h)

The sonicated sample of Pepperbark was indicated by 100% of the untrained tasting panel as the more intense in terms of flavour extracted, agreeing with the chromatographic peak intensity in Figure 3.7, above.

It can be seen in the resulting chromatogram, Figure 3.8, below, that although less intense than the sonicated sample chromatogram (Figure 3.7), the peak intensity of the sample stirred for 3 hours resulted in the greatest intensity, followed by the 6-hour and lastly the 24-hour sample (Figure 3.8).

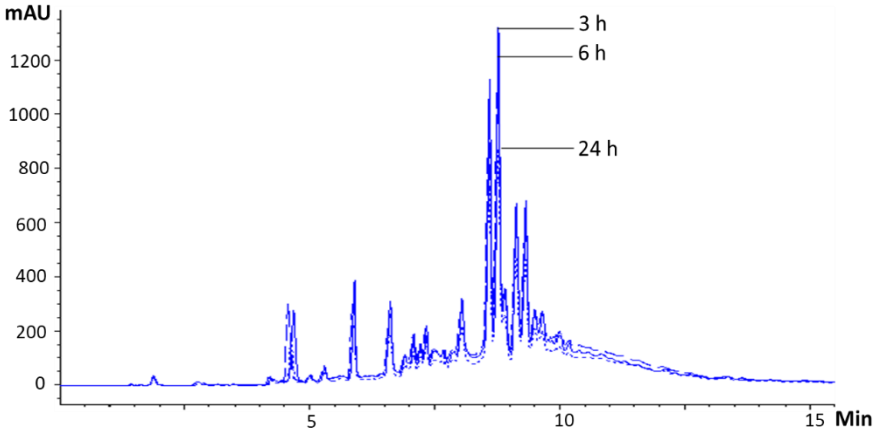

Figure 3.8 Chromatograms (254 nm) comparing the peak intensities of *Warburgia salutaris* samples stirred in a water bath at 70°C for different times

The sample stirred for 3 h yielded the greatest taste intensity out of the stirred samples, indicated by the 83% of the untrained sensory panel, agreeing with the peak intensity in the chromatogram generated (Figure 3.8).

3.2 Discussion of extraction conditions results

The solid–liquid extraction applied to the botanicals in this study represents a common way to isolate phytochemicals from raw plant-based products (Nastic *et al*., 2018; Anbalagan *et al.*, 2019; Zhang *et al.*, 2019). The traditional techniques for extracting phytochemical ingredients from plant cells are based on the augmentation of solvent effect with the aid of physical force and/or heat (Anbalagan *et al.,* 2019). The sample is generally ground to a fine powder to increase the specific surface area of the sample to enhance solvent–particle contact as well as mass transfer in the form of diffusion (Pronyk & Mazza, 2009; Anbalagan *et al.*, 2019; Zhang *et al.*, 2019). Plant cell walls are damaged during size reduction of the matrix particles, improving solvent penetration and product extraction (Pronyk & Mazza, 2009).

The solvents used for an extraction influence the nature and quantity of secondary metabolites extracted from the base plant material, as was discovered in the tasting outcome of the botanical extracts produced by using different concentrations of ethanol in the aqueous solvent (Dirar *et al.*, 2019). Since the general principle for solvent choice is that “like dissolves like”, polar solvents, such as water and ethanol, are used to extract hydrophilic compounds such as phenolic compounds, their glycosides and saponins, while non-polar solvents are used for the extraction of hydrophobic fatty acids and steroids (Mustafa & Turner, 2011; Dirar *et al.*, 2019). Numerous studies have been devoted to the impact of different solvent systems on the extraction of secondary metabolites and/or their antioxidant capacity (Belwal *et al.,* 2018; Dirar *et al.*, 2019; Arina & Harisun, 2019). The traditional solvent choice for the extraction of phytochemicals from plant matrices includes organic solvents and their mixtures in combination with water; these organic solvents include acetone, methanol, ethyl lactate, and chloroform (Zhang *et al.*, 2019). Because hydroalcoholic mixtures of ethanol and water present a suitable solvent system for human consumption, this solvent system was selected for the present investigation (Durling *et al*., 2007).

The solubility characteristic of the target analytes, their diffusivity in the solvent and the characteristics of the sample are all determining factors in solvent selection (Mustafa & Turner, 2011). Because the intention was to extract unspecified, maximum flavour from every botanical, no specific secondary metabolites were targeted and therefore extracts prepared using different ratios of water and ethanol for each botanical were tasted and it was decided by an informal, untrained sensory panel that the 50% aqueous ethanol ($v.v^{-1}$) mixture was responsible for extracting the preferred taste and flavour from each botanical.

It has been shown that dual solvent mixtures, for example the applied 50% aqueous ethanol solvent ($v.v^{-1}$), potentially enhance extraction yields by improving the solubility and increasing interaction of the analytes with the extraction solvent (Mustafa & Turner, 2011; Anbalagan *et al.*, 2019). In a solvent mixture, one solvent could improve the analyte solubility

while the other might enhance desorption (Mustafa & Turner, 2011). Water usually facilitates the breaking of matrix and matrix–analyte (hydrogen) bonds (Mustafa & Turner, 2011).

In a review article by Azmir *et al*. (2013), it was reported that water extracts anthocyanins, tannins, saponins and glycosylated terpenoids while ethanol extracts tannins, polyphenols, flavonols, terpenoids and alkaloids (Azmir *et al*., 2013). The combination of the two therefore covers all three categories of bioactive compounds in plants, namely terpenes and terpenoids, alkaloids and phenolic compounds (Azmir *et al.*, 2013).

Water is the more polar of the two solvents thus the addition of ethanol lowers the dielectric constant of the aqueous solvent, increasing the diffusion of molecules in the solvent (Bamba *et al.*, 2018). In a study of the extraction of bioactive phenolics from blueberry pomace, Bamba *et al*., (2018) found that a concentration of 50% ethanol ($v.v^{-1}$) was the most effective for extracting total phenolics, flavonoids and anthocyanins from the plant-based sample matrix. Similarly, 50% ethanol ($v.v^{-1}$) was reported as the most effective solvent system for the extraction of bioactive compounds from white pine needles (*Pinus morrisonicola*) (Chiang *et al.*, 2017). Deviations in these results from other work confirm that the efficiency of secondary metabolite extraction in ethanolic solvents is greatly influenced by variables including the plant material and extraction conditions (Durling *et al*., 2007; Bamba *et al.*, 2018). Despite the reported variations, most studies indicate that when concentrations of ethanol are too high or too low, the aqueous solvent becomes less efficient for the simultaneous extraction of phenolic compounds (Durling *et al*., 2007; Chiang *et al.*, 2017). Ethanol concentrations between 50% and 90% ($v.v^{-1}$) could also be effective, however, 50% ($v.v^{-1}$) seems a good compromise to cut costs and to keep the extraction method as environmentally friendly as possible (Durling *et al*., 2007; Chiang *et al.*, 2017; Bamba *et al.*, 2018).

Additional considerations affecting solvent choice includes economical aspects, safety, and sustainability - the move towards the use of “green” solvents aims to minimise the harmful effects of solvents on the environment (Mustafa & Turner, 2011). Examples of green solvents include water, of course, as well as simple alcohols, such as ethanol and methanol and the alkanes, heptane and hexane (Mustafa & Turner, 2011). Naturally, when solvents are combined with water in dual solvent systems, the environmental impact is further reduced (Mustafa & Turner, 2011).

The solid-liquid ratio is another important factor that affects the extraction efficiency (Zhang *et al*., 2019). While an excessive solid-liquid ratio can lead to solute saturation, small solids concentrations may increase the solvent cost (Zhang *et al*., 2019). In the current experiment, the solid-liquid ratio was not optimised per botanical. Instead, the solids concentration was maximised in for every extract, while maintaining a fluid mixture. Although concentrated extracts were prepared, there is a likelihood the solid-liquid ratio was unnecessarily large and that the extracts became saturated (Zhang *et al*., 2019).

Agitation during extraction facilitates the diffusion of analytes from the sample to the extraction medium, thereby enhancing extraction efficiency (Anbalagan *et al.*, 2019; Zhang *et al.*, 2019). When comparing the extraction techniques, namely sonication and stirring, for 3 h at 70°C, with constant solvent concentrations and solid-liquid ratios for each botanical, stirring proved to be the more efficient extraction technique for Honeybush, Moringa and Baobab, while sonication proved to be the more efficient of the two methods for Pepperbark. The decisions were made based on peak intensities in the chromatograms obtained, Figure 3.1 (Baobab), Figure 3.3 (Honeybush), Figure 3.5 (Moringa) and Figure 3.7 (Pepperbark), as well as the flavour intensity of the extracts as decided by the untrained sensory panel.

The alternative extraction technique, ultrasound-assisted extraction (UAE), or sonication, is based on the application of ultrasonic energy as a means of agitation of the matrix particles to be extracted, facilitating the leaching of organic and inorganic compounds from a plant matrix (Vintatoru, 2001; Rostagno *et al.*, 2003; Azmir *et al*., 2013; José & Pérez, 2019). The extraction mechanism by ultrasound involves two main types of physical phenomena, firstly, the diffusion across the cell wall and secondly, rinsing the contents of cell after breaking the walls (Azmir *et al*., 2013).

The sound waves from ultrasound are not detectable to the human ear and typically range from 20 kHz to 100 MHz (Azmir *et al*., 2013). When the ultrasonic radiation passes through a medium, a periodic disturbance is created, giving rise to expansion and compression cycles, introducing a phenomenon called cavitation, a term referring to the production, growth and collapse of bubbles (Rostagno *et al*., 2003; Azmir *et al*., 2013; José & Pérez, 2019). Bubble implosion creates changes in the temperature and pressure, enhancing the penetration of the solvent into the matrix, and increasing the contact surface area between the solid and liquid phase (Vintatoru, 2001; Rostagno *et al*., 2003; Azmir *et al*., 2013; José & Pérez, 2019). In turn, the mass transfer of the analytes into the solvent is enhanced (Vintatoru, 2001; Rostagno *et al.*, 2003; Azmir *et al*., 2013; José & Pérez, 2019). In addition, the use of elevated temperatures in UAE can increase the efficiency of the extraction process through the increased number of cavitation bubbles formed (Rostagno *et al*., 2003). UAE is considered an environmentally friendly extraction technique due to reduced extraction times, energy consumption and solvent usage compared to conventional extraction techniques (Azmir *et al*., 2013; José & Pérez, 2019). Sonication facilitates more effective mixing, faster energy transfer, reduced thermal gradients and extraction temperature when thermally labile constituents need to be extracted (Vintatoru, 2001; Rostagno *et al*., 2003; Azmir *et al.*, 2013; Trojanowska *et al*., 2019). The advantages of UAE over conventional solid-liquid extraction make it ideal for application in plant-based chemistry (Azmir *et al.*, 2013; Trojanowska *et al*., 2019).

The advantages of sonication over conventional extraction techniques are dependent on the UAE parameters; these factors include the solvent choice, irradiation conditions

(temperature and amplitude of sonication), as well as sonication time, sample particle size, solid-liquid ratio and radiation source (Azmir *et al.*, 2013; José & Pérez, 2019). Ultrasonic energy can be supplied by an ultrasonic water bath or by devices, such as probes, sonoreactors or microplate horns (José & Pérez, 2019). The most common and affordable of these is the ultrasonic bath, which allows the simultaneous extraction of several samples (José & Pérez, 2019).

Since sonication for 3 h at 70°C yielded the best extraction of flavour from Pepperbark (Figure 3.7), perhaps a lower temperature and/or shorter time could improve the extraction efficiency even further.

Multiple studies have shown UAE to be an effective extraction technique for secondary metabolite extraction from herbal materials (Azmir *et al.*, 2013). For example, in 2015, Li *et al.*, found that UAE of *Eucommia ulmodies* leaves at optimised extraction conditions improved the extraction efficiency of chlorogenic acid compared to traditional methods. In the review article by Azmir *et al.,* (2013), numerous studies reporting enhanced extraction efficiency of bioactive components from plant-based matrices by UAE are discussed.

Maceration with magnetic stirring is considered a conventional extraction technique and involves a magnetic stirrer as a source of agitation, where an external heat source, for example a heated water bath or hot plate, can be implemented (Nabarlatz *et al.*, 2010; Vieitez *et al.,* 2018). In a study of extraction of isoflavones from soybeans, Rostango *et al.*, (2003), showed that in different solvent compositions, the total and individual isoflavone yields obtained by UAE were generally 0-15% higher than those obtained with magnetic stirring.

In contrast, maceration with magnetic stirring proved to be the more efficient extraction technique compared to UAE and MAE, for the extraction of bioactive compounds from *Arbutus unedo* fruits (Albuquerque *et al.*, 2018). Extraction variables were optimised for each extraction technique using the response surface methodology method (RSM) and the efficiency was based on the preservative potential of the phytochemicals extracted (Albuquerque *et al.*, 2018). Regarding extract composition, in terms of extraction yield of the residue material extracted, the total carbohydrate content, the total phenolic content (TPC) and total flavonoid content (TFC), only slight differences were detected among the techniques, however, maceration proved to be the best technique, followed by UAE and MAE (Albuquerque *et al.*, 2018). Based on preservative potential, the macerated extracts resulted in extracts showing higher activities for the ABTS (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) and CM (Crocin method) assays, while the extracts obtained by MAE showed better activity based on the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and βCM (β-carotene bleaching inhibition method) assays (Albuquerque *et al.*, 2018).

Different extraction methods offer alternatives to attain different outcomes, resulting in flexible solutions for industrial purposes (Albuquerque *et al.*, 2018). Processing parameters

should be optimised for each extraction technique to produce extracts with the desired properties (Albuquerque *et al.*, 2018).

The effect of the duration of extraction under elevated temperature (70°C) on the flavour intensity and chromatographic peak intensity were assessed when the solid-liquid ratio and temperature (70°C) were kept constant for each botanical, stirring each of them in a water bath at 3 h, 6 h and 24 h (Figure 3.2, 3.4, 3.6 & 3.8). In the case of Honeybush (Figure 3.4), the peak intensity remained similar throughout, however, the majority of the higher peaks belonged to the sample stirred for 24 h. The informal taste results of Honeybush flavour intensity, determined by the untrained sensory panel, corresponded to the peak intensities recorded. Since Honeybush is generally extracted via hot water extraction using boiling water, it is expected that the compounds responsible for flavour in Honeybush tea are not highly sensitive to thermal degradation (Erasmus *et al.,* 2017).

In contrast, the taste- and peak intensities in the chromatograms of Pepperbark, and Moringa, decreased with increasing time under elevated temperature (70°C) (Figure 3.8 & 3.6). Three hours and 6 h resulted in similar taste and peak intensities for Baobab, however 24 h stirring was the least efficient time (Figure 3.2). This outcome suggests that the compounds responsible for the taste of the botanical samples are susceptible to thermal degradation.

Temperature is an important parameter that can be manipulated to enhance extraction efficiency (Ramos, Kristenson & Brinkman, 2002; Mustafa & Turner, 2011; Chiang *et al.*, 2017; Nastic *et al*., 2018; Arina & Harisun, 2019). It has been reported that the application of high temperature during extraction decreases the dielectric constant (polarity) of water, making it a suitable solvent to extract polar, moderately polar and non-polar organic compounds (Ramos, Kristenson & Brinkman, 2002; Mustafa & Turner, 2011; Ong *et al.*, 2006; Nastic *et al*., 2018). Elevated temperature is also an effective way to overcome analyte-matrix interactions caused by van der Waals forces, hydrogen bonding and dipole attraction, thereby improving extraction efficiency (Ramos, Kristenson & Brinkman, 2002; Mustafa & Turner, 2011; Chiang *et al.*, 2017). By applying thermal energy, cohesive and adhesive interactions between analytes and the matrix material are disrupted, by decreasing the activation energy required for the desorption process (Mustafa & Turner, 2011; Chiang *et al.*, 2017). Elevated temperature further decreases the surface tension of the solvent, solutes and matrix, enhancing the solvent wetting of the sample (Ramos, Kristenson & Brinkman, 2002; Mustafa & Turner, 2011; Chiang *et al.*, 2017; Nastic *et al*., 2018). Decreased solvent surface tension allows solvent cavities to be formed more easily, thus permitting analytes to faster dissolve in the solvent (Ramos, Kristenson & Brinkman, 2002; Mustafa & Turner, 2011; Chiang *et al.*, 2017; Nastic *et al*., 2018). The decreased solvent viscosity of a liquid solvent associated with an increase in temperature enhances its penetration inside the matrix particle, resulting in an enhanced

extraction (Ramos, Kristenson & Brinkman, 2002; Mustafa & Turner, 2011; Nastic *et al*., 2018). Another advantage of using higher temperature of the solvent is the improved diffusion rate, enabling faster extractions especially in diffusion-controlled samples (Ramos, Kristenson & Brinkman, 2002; Mustafa & Turner, 2011). In other words, temperature affects the selectivity as well as the efficacy of an extraction (Ramos, Kristenson & Brinkman, 2002; Mustafa & Turner, 2011; Nastic *et al*., 2018).

A known drawback of high temperature extraction is that prolonged exposure potentially has a detrimental effect on plant extracts, causing loss in quality and biological activity due to hydrolytic and thermal degradation of thermo-labile constituents (Ramos, Kristenson & Brinkman, 2002; Mustafa & Turner, 2011; Ong *et al*., 2006; Chiang *et al.*, 2017; Anbalagan *et al.,* 2019).

In 2017, Chiang *et al*. experimented with the effect of extraction temperature on the TFC and TPC extracted from white pine needles (*Pinus morrisonicola*). Extractions were conducted at 40, 50, 60, 70, 80 and 90°C, with 40 $mL.g^{-1}$ of liquid–solid ratio, 3 h of extraction time with 30% $v.v^{-1}$ aqueous ethanol as solvent (Chiang *et al.*, 2017). Increased extraction yields were obtained for both TFC and TPC from 40°C to 70°C (Chiang *et al.*, 2017). Above 80°C, the extract TPC increases with temperature, however, the TFC equivalent decreases between the temperatures, 70°C and 80°C (Chiang *et al.*, 2017). These results were confirmed by the increasing antioxidant activity with the increasing extraction temperature up to 70°C, after which a decline was observed (Chiang *et al.,* 2017). The decrease in TFC above 70°C was attributed to the thermal degradation and oxidation of compounds such as anthocyanins, which in turn decreases the antioxidant activity of the extracts at high temperature (Chiang *et al.*, 2017).

Similarly, an investigation of extraction parameters on extraction efficiency of phenolic compounds with antioxidant activity from the Brazilian shrub, *Baccharis dracunculifolia,* found that increasing the extraction temperature from 40°C to 80°C, increased the extraction of bioactive compounds by approximately 100%, based on the DPPH, ABTS, and FRAP (ferric reducing ability of plasma) assays (Casagrande *et al*., 2018).

Gullón *et al.* (2017) determined the optimum conditions for the extraction of bioactive compounds from eucalyptus leaves *(Eucalyptus globulus),* with the highest experimental temperature being 50°C. The highest extraction was achieved for the sample extracted at 50°C, for 225 min with an ethanol concentration of 56% ($v.v^{-1}$) (Gullón *et al.*, 2017). In a follow up study, higher temperatures, 60°C and 70°C were also investigated, and although the TPC and TFC remained constant, the antioxidant activity decreased (Gullón *et al.*, 2017).

Thus, the selection criteria of a suitable extraction temperature should consider both the extraction efficiency and thermal stability of the active compounds (Chan, Yusoff & Ngoh, 2014; Nastic *et al.,* 2018).

3.3 LC Taste® fractionation

For each of the chromatograms illustrated below (Fig. 3.9-3.12), the red block encloses the fraction-collection window, while the two diagonal lines represent the solvent gradient (green – ethanol; pink – water).

The chromatograms are provided on a normalised scale.

3.3.1 *Adansonia digitata*

Figure 3.9 below shows the LC Taste® fractionation of the 25% solids (w.v^{-1}) Baobab solution, magnetically stirred in a water bath at 70°C for 3 h. The overlapping chromatograms represent the duplicate runs (254 nm).

The overriding taste of each of the fractions tasted, was sour, as described by the untrained panel.

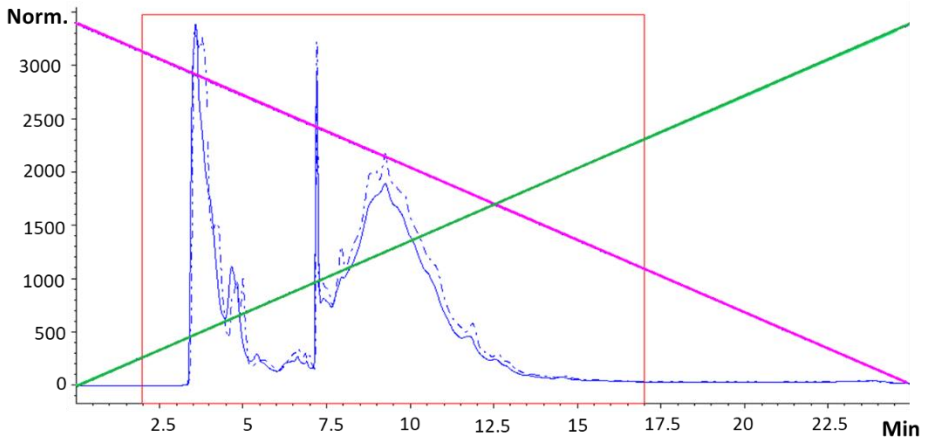

Figure 3.9 Chromatograms (254 nm) indicating the LC Taste® separation of the Baobab extract

3.3.2 *Cyclopia genistoides*

Figure 3.10 below shows the LC Taste® fractionation of the 25% solids (w.v^{-1}) Honeybush solution, magnetically stirred in a water bath at 70°C for 24 h.

The general taste detected for fractions 4 to 8 was sour, fractions 9 to 12 were sweet in taste and 13 to 15 had an unfamiliar taste. Thereafter, the ethanol content became too high to identify possible underlying tastes and/or flavours in the fractions.

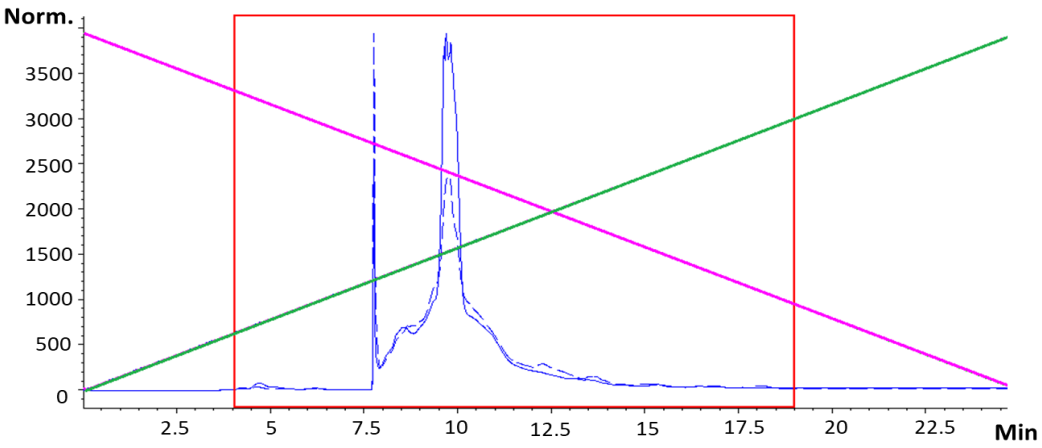

Figure 3.10 Chromatograms (254 nm and 280 nm) indicating the LC Taste® separation of the Honeybush extract

3.3.3 *Moringa oleifera*

Figure 3.11 below illustrates the LC Taste® fractionation of the 15% solids (w.v^{-1}) Moringa solution, magnetically stirred in a water bath at 70°C for 3 h.

Moringa fractions 3 to 8 were sour, 9 to 10 were unfamiliar, while 11 to 16 were predominantly bitter in taste, as described by the untrained sensory panel. Fractions 17 and 18 were too high in ethanol to identify underlying flavours and/or tastes.

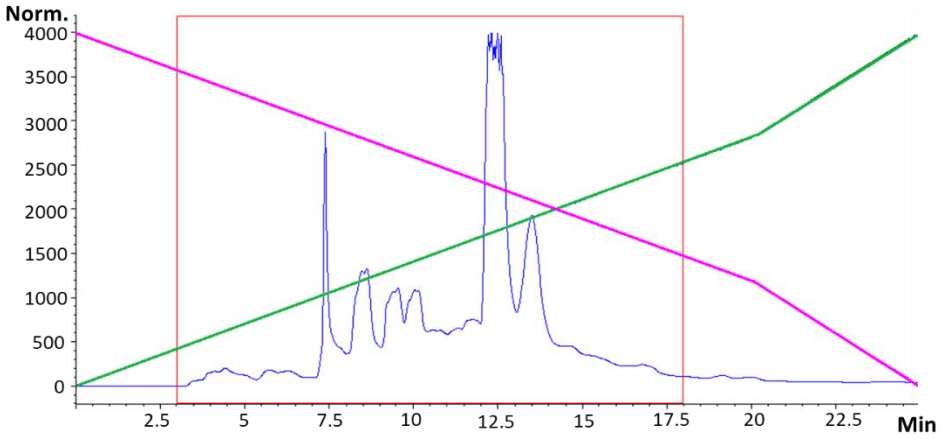

Figure 3.11 Chromatogram (280 nm) indicating the LC Taste® separation of the Moringa extract

3.3.4 *Warburgia salutaris*

Figure 3.12 below represents the LC Taste® fractionation of the 25% solids (w.v^{-1}) Pepperbark solution, sonicated at 70°C for 3 h.

Fractions 7 to 9 were sour, while fractions 10 and 11 had a characteristic peppery taste, leaving a tingling sensation on the lips and tongue after tasting, as described by the

untrained sensory panel. Fraction 12 and 13 were bitter. The ethanol content became too high to identify underlying tastes and/or flavours in the remaining fractions.

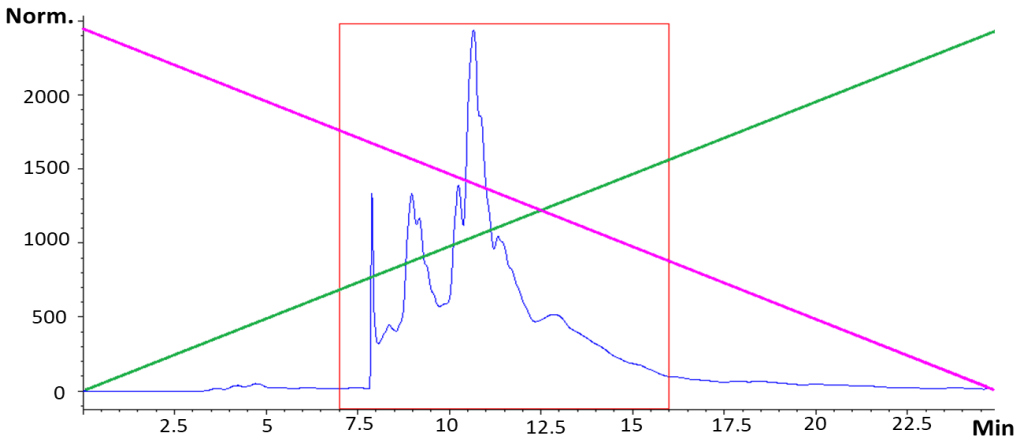

Figure 3.12 Chromatogram (254 nm) indicating the LC Taste® separation of the Pepperbark extract

3.3.5 Sensory feedback from Kerry Foods

After tasting the eluted LC Taste® fractions of all four botanicals, Kerry Foods' expert sensory panel was most interested in fraction 11 from *Warburgia salutaris*. It was described by the expert tasters as having a pungent taste and a distinctive, lasting, tingling sensation in the mouth (Dovey, M. 2019, Technical Manager, Kerry Ingredients and Flavours, Durban, South Africa, personal communication, 17 November). The sensory panel accepted fraction 11 to be subjected to further analysis.

3.4 Discussion of the LC Taste® results

Once suitably concentrated extracts were prepared for each botanical, LC Taste® enabled the sensory panels, both preliminary and expert, to taste the collected fractions from each the botanicals separately. Because the extracted phytochemicals from each botanical were separated by the mobile phase gradient in HPLC, the flavours and tastes associated with the phytochemicals were also separated in each of the botanicals and could be tasted individually or in less complex fractions, without dominant flavours and tastes, such as bitterness or sourness, overpowering the underlying flavours unique to each botanical.

A very large injection volume of 1 mL was used to ensure that sufficient flavour would be detected in the eluted fractions, while a high mobile phase flow rate of 3 mL was selected for the method to ensure that sufficient fraction volumes could be collected for the sensory evaluation of the fractions; both these parameters are commensurate with the polymeric column dimensions. Due to the large injection volume and the short analysis times selected to reduce the number of fractions for tasting and the overall analysis time, relatively unresolved

chromatograms were obtained – although these parameters can be further optimised to improve peak separation, the aim of LC Taste®, to rapidly screen complex extracts for interesting flavours/tastes, was achieved (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c).

The fraction collection intervals, enclosed in red blocks in each of the chromatograms (Figures 3.9 – 3.12), were selected based on ultraviolet (UV) signals recorded at 254 nm and 280 nm. Many secondary metabolites have conjugated systems in their structures and are thus easily detected using UV spectroscopy (Sisa *et al*., 2010). The wavelengths selected in this study are two commonly selected wavelengths used to detect aromatic compounds and phenolics in organic samples, including wine and a range of medicinal plants (Hosseinian *et al.*, 2008; Sisa *et al*., 2010; Aleixandre-Tudo & du Toit, 2018).

It was found that where prominent peaks were present in the chromatograms, flavours/tastes, other than sour, were detected, provided that the ethanol concentration was not too high. The first set of fractions tasted for each botanical, where no prominent peaks were visible, were sour in taste. The likely compounds responsible are low molecular weight organic acids that are water soluble and thus elute in the predominantly aqueous part of the water-ethanol gradient (Wang *et al*., 2018). Furthermore, low molecular weight organic acids typically absorb UV light at lower wavelengths (210 nm) than the selected wavelengths (da Costa *et al.,* 2016). The *Adansonia digitata* fractions were all sour, with varying intensities. In the case of *Moringa oleifera, Warburgia salutaris* and *Cyclopia genistoides*, the fractions that eluted in in the middle region of the gradient, ranging from approximately 30% to 55% ethanol ($v.v^{-1}$), were the most pleasant tasting. Unique flavours from each botanical could be tasted in this region, correlating with the previously reported result that the 50% ethanol ($v.v^{-1}$) extracted the preferred taste/flavour profile from each of the botanical extracts initially. As previously discussed in the selection of a suitable extraction solvent, many plant secondary metabolites are soluble in 50% aqueous ethanol and therefore many phytochemicals, including phenolic compounds, anthocyanins and flavonoids would be eluted in this region of the water-ethanol gradient (Durling *et al*., 2007; Chiang *et al.*, 2017). At higher concentrations of ethanol, no tastes were detected due to the domineering taste of the alcohol.

LC Taste® successfully enabled the rapid screening of fractions for tastes and flavours present in a variety of botanicals and also gave a rough indication of the solubility properties of the compounds, based on where in the water-ethanol gradient they eluted. There was no need for subsequent steps to remove harmful solvents and fractions could be tasted in the form in which they were collected (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c).

Further investigation of fractions is required to identify the specific compounds responsible for the perceived sensory properties (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c).

3.5 Microbial results and discussion

Before sensory evaluation was done on the botanical fractions, the microbial outcome indicated that no growth was present on either the XLD plates, nor on the MYP plates for any of the four botanicals, after primary and secondary examination of the microbial plates, suggesting that the final fractions that are safe for human tasting.

The extraction conditions as well as the LC Taste® fraction collection procedure of each of the botanicals involves sequential steps of heating and ethanol treatment, both which are well-known microbial hurdles (Casadei *et al.*, 2001; Møretrø *et al*., 2012; He *et al*., 2018; Cui *et al*., 2019).

Salmonella spp. are non-spore-forming, enteric, thermo-sensitive microbes (Graziani *et al*., 2017). In the food industry, proper hygienic practice normally keeps *Salmonella* prevalence at a low level, but breach in routines may lead to *Salmonella* contamination on equipment or other surfaces, which in turn may transfer to the food via cross-contamination (Møretrø *et al*., 2012). *Salmonella* spp. grow in the temperature range, 5–47°C, with the optimal temperature ranging from 35–37°C and are generally destroyed at temperatures equal to, or greater than 70°C (ICMSF, 1996; Graziani *et al*., 2017; Cui *et al.,* 2019).

However, as with most microbes, the heat resistance of *Salmonella* spp. in food is largely dependent on the composition, pH and water activity of the food (Graziani *et al*., 2017; Cui *et al*., 2019). As the water activity of the food decreases, the heat resistance of *Salmonella* spp. increases - foods that are high in fat and low in moisture, such as chocolate and peanut butter, may have a protective effect against heat (Graziani *et al*., 2017; Cui *et al*., 2019). The optimum water activity for *Salmonella* spp. is 0.99, with the lower limit for growth at 0.93 (Cui *et al.,* 2019). *Salmonella* spp. can survive in water activities as low as 0.20 or lower and can survive for months, or even years, in dry products, such as black pepper and gelatine (ICMSF, 1996; Podolak *et al.* 2010; Graziani *et al*., 2017; Cui *et al*., 2019).

Ethanol is an effective disinfection agent against *Salmonella* spp., usually at 70%, although under certain conditions, lower concentrations have also shown to be effective (Møretrø *et al*., 2012; He *et al*., 2018). The mechanism by which ethanol kills microorganisms is through membrane damage and denaturation of proteins (Møretrø *et al*., 2012; He *et al*., 2018). It is therefore almost certain that during the extraction and fraction collection procedures, *Salmonella* spp. in the botanical extracts will be destroyed.

Bacillus cereus is ubiquitous in nature (Jessberger *et al.,* 2019). Its prevalence in soil and crops, together with its ability to form highly resistant endospores, makes contamination

during food production and processing challenging to avoid (Jessberger *et al.,* 2019). Moreover, this gram-positive bacterium is highly resistant to harsh conditions through its formation of biofilms and spores, justifying the great concern towards this pathogen in the food industry (Lv *et al.*, 2019). The spores remain metabolically inactive in the environment but take the form of vegetative cells when they infect the human body (Xaplanteri *et al.*, 2019). The most common source of *B. cereus* food poisoning is caused by cooked starchy foods, such as cooked rice, cereals, and beans, that are not properly cooled or held hot after cooking (Hwang & Huang, 2019).

In 2019, Hwang and Huang used a one-step dynamic analysis to construct a tertiary model to describe the growth and survival of *B. cereus* and estimate the kinetic parameters in starch-based products. The minimum, optimum, and maximum growth temperatures were shown to be 8.2, 37.6, and 46.8°C, respectively (Hwang & Huang, 2019). It was also found that populations of *B. cereus* decreased gradually at the rate of 1.21×10^{-3} ln CFU/g/h per °C below the minimum growth temperature (Hwang & Huang, 2019).

Spores produced by *Bacillus cereus* are very tenacious and withstand elevated temperature, toxic chemicals, UV rays, γ-rays and other unfavourable environments (Jiao *et al.,* 2019; Shu *et al.,* 2019). The spores of *B. cereus* cannot be killed by usual food heating and cooking and remain in food to germinate (Gonzalez *et al.*, 1999; Shu *et al.,* 2019).

The thermal inactivation of *B. cereus* spores is influenced by multiple factors, including the sporulation temperature, the composition and pH of the heating medium (Gonzalez *et al.*, 1999; Moussa-Boudjemaa *et al*., 2006). In 2001, Casadei *et al.* studied the combined effect of pH and ethanol on the heat resistance of *B. cereus* spores. It was found that increasing the amount of ethanol to 10% decreased the *D* values (decimal reduction dose) by approximately two-fold at a neutral pH, and by approximately three-fold at pH 5 and 3 (Casadei *et al.*, 2001). The adverse effect of ethanol on the heat resistance of *B. cereus* was independent of treatment temperature (Casadei *et al.*, 2001). This study indicates that the combination of ethanol and heat, as applied in the present study, is effective in reducing and/or destroying *B. cereus* spores in food.

Concerning the initial microbial quality of the botanicals, the fruit pulp of *Adansonia digitata* is a considered a microbially stable product, with the naturally dehydrated, low-moisture content (10-12%) of the ripe fruit, as well as the acidic nature of the pulp, with a pH value of around 3.3 (Chadare *et al*., 2009; UNCTAD, 2005). As with the other botanicals at hand, Baobab fruit pulp is also known for its antibacterial properties, presenting yet another microbial hurdle to certain microbes (Kamatou *et al.*, 2011; Ismail *et al*., 2019). The antimicrobial properties of Baobab fruit pulp are attributed to the presence of tannins, phlobatannins, terpenoids and saponins (Kamatou *et al.*, 2011; Ismail *et al*., 2019). In the case of wild-harvested Baobab fruit, these may be subjected to conditions that favour mould growth,

and it has consequently been suggested by the EFSA that the fruit pulp should be tested for the presence of aflatoxins (Hermann, 2009). Aflatoxins are mycotoxins produced primarily by *Aspergillus* species and are considered among the most challenging classes of mycotoxins contaminating a wide range of foodstuffs and agricultural products during growth, harvest, storage, and transportation (Kademi *et al*., 2019).

Investigation of sorption isotherm behaviour of *M. oleifera* leaf powder showed an increase in moisture content with the increase of water activity and the curve was characterised by a sigmoidal form, similar to the behaviour of other medicinal and aromatic plants (Rébufa, Pany & Bombarda, 2018). For water activity values below 0.5, the content of available free water was almost constant (8–10%), indicating that *M. oleifera* dried leaf powder is microbiologically stable and consequently has an extended shelf-life (Rébufa, Pany & Bombarda, 2018).

While the leafy shoots and flowers of Honeybush were traditionally fermented and sun-dried in heaps to prepare tea, modern, commercialised production of fermented tea allows far more control over production parameters (Joubert *et al*., 2008; Joubert *et al*., 2011; Stepanova *et al*., 2012). The increasingly high demand for high quality tea and strict export regulations regarding microbial contamination have led to improved processing techniques (Joubert *et al*., 2008). Commercially, the plant material is mechanically cut into small pieces and moistened to approximately 65% moisture content before being fermented (80°C for 24 h or 90°C for 16 h) and dried under controlled conditions (40°C for 6 h) (Joubert *et al.*, 2008; Theron *et al.,* 2014). Not only does the high temperature fermentation and drying under controlled conditions improve the sensory quality of the tea, temperatures ≥60°C delivers a product with very low total bacterial counts (<1 000.g^{-1}), with no moulds, *Salmonella or Escherichia coli* present (Joubert *et al*., 2008; Joubert *et al.*, 2011).

Although no articles have been devoted to the microbial risks associated with *Warburgia salutaris,* raw materials for botanical preparations, including pepperbark leaves, are generally traded in dried form (Warude & Patwardhan, 2004; EHIA, 2008; Fogele *et al*., 2018; Székács *et al.,* 2018; Thanh *et al*., 2018). These low moisture products are mainly at risk of bacterial endospores and fungal spore contamination, capable of surviving low humidity conditions (Warude & Patwardhan, 2004; EHIA, 2008; Fogele *et al*., 2018; Székács *et al.,* 2018; Thanh *et al*., 2018).

4. CONCLUSION AND RECOMMENDATIONS

For LC Taste® fractionation to be successful, concentrated extracts are required. Concentrated extracts were prepared for each of the botanical matrices, namely *Adansonia digitata* (Baobab), *Cyclopia genistoides* (Honeybush), *Moringa oleifera* (Moringa) and *Warburgia salutaris* (Pepperbark) by manipulating multiple extraction parameters.

A hydroalcoholic solvent of aqueous ethanol was selected as a suitable extraction solvent that is safe for human consumption and a green solvent choice. The 50% ethanol concentration ($v.v^{-1}$) held the greatest potential in terms of the taste and flavour extracted from each botanical. Previous research work has indicated that a concentration of 50% ethanol ($v.v^{-1}$) is effective in extracting phytochemicals from the plant-based matrices.

Although maximising the solids concentration in the botanical extracts produced more concentrated extracts, the solid-liquid ratio is another important factor that affects the extraction efficiency. It is likely that in this study, an excessive solid-liquid ratio was used for the botanicals, which might have resulted in solute saturation. In future research, it is recommended that the solid-liquid ratio should be further optimised per botanical.

When comparing the extraction techniques evaluated in this study, magnetic stirring led to better extraction of flavour from Honeybush, Moringa and Baobab, while sonication resulted in better flavour intensity in the case of Pepperbark. Both techniques offer a range of advantages that make them suited for extraction of phytochemicals from botanical sources. Processing parameters must however be optimised for each extraction technique to produce food extracts with the desired properties.

The effect of the extraction time at elevated temperature (70°C) on the taste and peak intensities of the chromatograms indicated that 24 h was the best for flavour extraction from Honeybush. This suggests that the compounds responsible for flavour in Honeybush tea are not highly susceptible to thermal degradation.

In contrast, the taste- and peak intensities of Pepperbark and Moringa extracts decreased with increasing time at elevated temperature (70°C). Three hours and 6 h resulted in similar taste and peak intensities for Baobab, however 24 h stirring was the least efficient time. This outcome suggests that the compounds responsible for the taste of the botanical samples may be relatively susceptible to thermal degradation or alteration. In follow-up research, it would be valuable to experiment with a wider temperature range, using both UAE and magnetic stirring to study the effect on the flavour extracted from various botanical sources.

Once concentrated extracts of the preferred flavour were prepared for each botanical, LC Taste® proved to be a useful method for screening the flavours and tastes present in each of the botanicals, without dominant bitter or sourness overpowering the underlying flavours unique to each botanical. A large injection volume and high mobile phase flow rate, in addition to a short analysis time, were selected to ensure that sufficient fraction volumes with detectable flavour could be collected for sensory evaluation in a timely manner. The choice of these parameters resulted in relatively poorly resolved chromatograms. Although these parameters can be improved to enhance the separation efficiency, the aim of LC Taste® - to rapidly screen complex extracts for interesting flavours/tastes - was successfully achieved.

As with any other ingredient intentionally added to food and beverage products, the microbial safety of botanical preparations is an important safety parameter to consider. The microbial results of this study indicated that the botanical fractions were all free from the pathogenic microbes, *Salmonella* spp. as well as *Bacillus cereus* spores, suggesting that the obtained fractions are microbiologically safe for human tasting. An additional microbiological test for at least one species of a toxigenic fungus is recommended in future work, for example *Aspergillus* spp., which is a common pathogen in botanical ingredients and is resistant to drying.

The *Adansonia digitata* fractions were all sour, with varying intensities, however, in the case of *Moringa oleifera, Warburgia salutaris* and *Cyclopia genistoides*, the fractions that eluted in in the middle region of the gradient, held the most flavour/taste potential – correlating with the previously reported result that the 50% ethanol solvent system ($v.v^{-1}$) extracted the preferred taste/flavour profile from each of the botanicals. At higher concentrations of ethanol, no tastes were detected, partially due to the overwhelming taste of the alcohol.

LC Taste® successfully enabled the rapid identification of flavour/taste-active fractions and gave an indication of the solubility properties of the compounds, based on where in the water-ethanol gradient they elute.

Further chromatography of fractions is required to identify the specific compounds responsible for the perceived sensory results. The outcome of this study agrees with existing literature that LC Taste® is a powerful screening tool, however, it is not intended to replace conventional fractionation, characterisation and the rigorous evaluation of interesting single compounds.

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CHAPTER 4

ANALYTICAL EVALUATION OF A PUNGENT FLAVOUR FRACTION FROM *WARBURGIA SALUTARIS* IDENTIFIED AND COLLECTED VIA LC TASTE® SCREENING

ABSTRACT

Warburgia salutaris is a highly sought-after medicinal plant species, recognised amongst the diverse southern African food plants for its potential as a novel flavour source. Its colloquial name, the “Pepperbark” tree, is descriptive of the fragrant, pungent, peppery taste of the leaves and bark. Using the flavour screening technique, LC Taste®, a pungent fraction of the leaf powder extract of *W. salutaris* that leaves a distinctive, tingling sensation in the mouth, was identified. To identify and quantify the compound(s) that contribute(s) to the characteristic flavour and associated heat detected in the fraction, qualitative and quantitative analyses were performed. Gas chromatography-mass spectrometry (GC-MS) was used to identify volatile compounds that may contribute to the aroma of the isolated fraction. Few aromatic compounds were detected since the collected fraction was relatively polar due the initial solvent system (50% aqueous ethanol; $v.v^{-1}$) used to extract the leaf powder as well as the water-ethanol gradient used in LC Taste®. Liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI-MS) and tandem mass spectrometry (MS/MS) analysis of the pungent Pepperbark fraction enabled the identification of the pungent piperine-type alkaloid, piperanine, as the most abundant compound in the fraction. The extraction of piperanine from *W. salutaris* leaf powder was performed based on parameters that are effective in the extraction of piperine from *Piper* fruit. The extraction technique, ultrasound-assisted extraction (UAE), the extraction temperature (50°C), and solid-solvent ratio (1:10) were kept constant while a variety of solvents (100% ethanol and 50% aqueous ethanol ($v.v^{-1}$); acidified aqueous solvents; pH 1.00, pH 4.00; pH 7.00) and different extraction times (18 minutes and 3 hours) were applied. The solvent, 50% aqueous ethanol, and an extraction time of 3 h proved to be the most efficient in the extraction of piperanine. Using a calibration curve of a piperanine standard, calculation of the piperanine content of *W. salutaris* suggested that 0.412% ($w.w^{-1}$) of the dried leaf powder constitutes the extracted compound. This quantity is subject to natural variation and further investigation could lead to improved extraction yields. Although piperanine was likely the sole contributor of pungency detected in the peppery fraction isolated from the *W. salutaris* extract, several hot-tasting compounds, sesquiterpenes, have been reported in *Warburgia* species. These were likely excluded by the initial extraction solvent comprising 50% aqueous ethanol ($v.v^{-1}$). Due to the growing market for functional foods and food and beverage products with natural flavours, colours and preservatives, extracts of *Warburgia salutaris* leaves with enhanced extraction yields of piperanine as well as pungent sesquiterpenoids, potentially hold great value, with attractive features including the novelty of

the flavour as well as the medicinal properties associated with piperanine and the sesquiterpenoids.

1. INTRODUCTION

In 2011, Van Wyk published a review article discussing the rich diversity of southern African food plants, as well as trends in the exploration and development of food and beverage products that incorporate these ingredients. In this review, *Warburgia salutaris* is mentioned as an aromatic plant with potential application as a novel flavour source (Van Wyk, 2011).

W. salutaris (Canellaceae) is distributed between the north eastern parts of South Africa, Swaziland, south-eastern Zimbabwe, southern Mozambique, Malawi and Zambia (Maroyi, 2013; Kotina *et al.,* 2014). It is a medium-sized, evergreen tree with fissured bark, simple, glossy leaves, green flowers and plum-shaped fruits (Kotina *et al.,* 2014).

Warburgia salutaris earned its colloquial name as the “Pepperbark” tree due to the fragrant, pungent, peppery taste of the leaves and bark (Leonard & Viljoen, 2015; Van Wyk & Prinsloo, 2019). Traditionally, the tree bark, as well as the fresh or dried leaves are used in various dishes to give them an aromaticity and a peppery taste (Kotina *et al.*, 2014; Leonard & Viljoen, 2015; Van Wyk & Prinsloo, 2019). The leaves have also been used to prepare a pleasant-tasting tea or as an appetite stimulant (Van Wyk & Prinsloo, 2019).

The near-panaceal properties of *W. salutaris*, were recognised at an early stage by the taxonomist who awarded the species with the epithet ‘‘salutaris’’ meaning ‘‘health giving’’ or ‘‘wholesome’’ (Van Wyk, 2008; Maroyi, 2013; Kotina *et al*., 2014; Leonard & Viljoen, 2015; Khumalo *et al*., 2019). The most common ailments treated with *Warburgia* species include gastro-intestinal, respiratory and odontological disorders, fever, malaria, colds, coughs and sore throats (Maroyi, 2013). *Warburgia* remedies are typically administered in the form of a decoction or infusion (54.5%), the bark can be chewed and juice swallowed (27.3%) or it can be applied in the form of an ointment or paste (10.4%) (Maroyi, 2013).

Because of the immense value of the *Warburgia* species in traditional African medicine, many researchers have studied the phytochemistry of the bark of these plants in an attempt to uncover the compounds responsible for its antibiotic and other pharmaceutical properties (Kotina *et al.,* 2014; Leonard & Viljoen, 2015). Because the bark of these trees is most frequently used in ethnomedicine, the chemical composition of the leaves of the species belonging to this genus is less familiar (Maroyi, 2013).

The use of the tree bark as a spice in east African cooking resulted in further investigation of the fragrant, often pungent, compounds (Leonard & Viljoen, 2015). One of the primary hot-tasting compounds isolated from *Warburgia* species was polygodial, previously isolated from the leaves of *Polygonum hydropiper,* called “water pepper” in Japan (Leonard & Viljoen, 2015). The hot taste of polygodial was attributed to the presence and configuration of the aldehyde functional group at C-9 of polygodial (Sterner and Szallasi, 1999). This aldehyde is in the β-configuration in polygodial, while it is in the α-configuration in its epimer, isopolygodial, which is tasteless (Sterner and Szallasi, 1999).

The unsaturated terpenoid dialdehydes contribute to the pungent taste in *Warburgia* species (Leonard & Viljioen, 2015; Szallasi *et al.,* 1998). Sterner and Szallasi (1999) found it interesting that the pungency detected by humans was associated with antifeedant properties in insects. In 1981, Kubo and Ganjian isolated the sesquiterpenes, polygodial, warburganal, muzigadial and ugandensidial from methanol extracts of the bark of the East African *Warburgia* trees, *Warburgia ugandensis* and *W. stuhlmannii.* These hot components, considered to be oxidation products of the drimenin skeleton, exhibit powerful antifeedant activity against the armyworms, *Spodoptera exempta* and *S. littoralis* as part of their natural defense mechanism (Kubo, 1995).

In 1999, Mashimbye *et al.* isolated the sesquiterpenes, warburganal, polygodial, mukaadial, isopolygadial as well as a new lactone drimane sesquiterpene, named salutarisolide, from the stem bark of a pepperbark tree from the Soutspanberg in the Limpopo province of South Africa. Rabe and van Staden (2000) isolated the drimane sesquiterpene muzigadial from *W. salutaris* for the first time. Drewes *et al.* (2001) used ^{1}H NMR to show that the ratio of the biologically active compounds, polygodial to warbuganal, are similar in the leaves and the bark, thereby offering a sustainable alternative to the stripping of the bark of this critically engendered species (Drewes *et al*., 2001; Kotina *et al*., 2014; Leonard & Viljoen, 2015; Van Wyk & Prinsloo, 2019).

A study of the essential oils from the bark and leaves of *W. salutaris* revealed the monoterpenes, myrcene, cis-β-ocimene and trans-β-ocimene as the major compounds in both the bark and leaves (Leonard & Viljoen, 2015). In addition, the sugar-alcohol, mannitol occurs in the bark of *W. salutaris* (Leonard & Viljoen, 2015). Mannitol is an osmotic diuretic agent and acts as an osmoregulator in the human body (Leonard & Viljoen, 2015). It may also be used as a sweetener for diabetics (Leonard & Viljoen, 2015).

While it is known that the leaves offer a potentially valuable alternative to the unsustainable practice of harvesting the bark based on the ratio the bioactive phytochemicals, polygodial and warburganal, limited research has been done on the remainder of the phytoconstituents present in the leaves of *W. salutaris.* To date, no research has been specifically devoted to the flavour potential of the leaves of *Warburgia* species leaves for food and beverage applications.

In Chapter 3 of this thesis, LC Taste® was used to screen a range of South African botanicals, namely *Adansonia digitata* (Baobab), *Cyclopia genistoides* (Honeybush), *Moringa oleifera* (Moringa) and *Warburgia salutaris* (Pepperbark), with the aim of identifying a fraction that holds potential as a flavour source for the food and beverage industry.

LC Taste® is a useful flavour screening method that enables the separation of non-volatile compounds in complex food matrices via reverse phase-high temperature liquid

chromatography (RP-HTLC), combined with direct sensory evaluation of eluted fractions (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c).

Because the eluted fractions must be safe for sensory analysis, a blend of non-toxic solvents is used, for example water and ethanol, typically used in combination with elevated temperature to reduce mobile phase viscosity to overcome pressure contraints (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c).

LC Taste® enables the screening of flavour fractions from complex solutions or extracts, without dominating flavours or tastes from other fractions interfering with the flavour or taste (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c). Once fractions of interest are identified, further analytical investigation can be used to identify the compounds responsible for the flavour detected (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c; Mittermeier *et al.*, 2018).

The fraction that gained the interest of the tasting panel was a fraction isolated from the leaf extract of *Warburgia salutaris*. It was described by the expert tasters as having a pungent taste with a distinctive, lasting, tingling sensation in the mouth (Dovey, M. 2019, Technical Manager, Kerry Ingredients and Flavours, Durban, South Africa, personal communication, 17 November).

The objective of the research reported in this chapter was to identify and quantify the compound(s) in the *W. salutaris* leaf powder extract that contribute(s) to the characteristic flavour and associated heat and tingling sensation detected in the isolated flavour fraction. To this end, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) analysis were used.

2. MATERIALS AND METHODS

2.1 Qualitative analysis of the *Warburgia salutaris* fraction of interest

2.1.1 Fraction collection

Samples of *Warburgia salutaris* (Pepperbark) dried leaf powder were obtained from Kerry Foods (Hillcrest, Durban, South Africa). The samples were stored in sealed containers in dry conditions at ambient temperature, out of direct sunlight.

As described in Chapter 3, the Pepperbark extract was prepared by sonicating (Hwashin Ultrasonic Cleaner, Power Sonic410, frequency 40 kHz, capacity 10 L, required power 400 W, Seoul, Korea) a 25% solids concentration ($w.v^{-1}$; 5 g.20 mL^{-1}) in 50% ($v.v^{-1}$) aqueous ethanol (≥99.9%, LiChrosolv, Sigma-Aldrich Merck, Darmstadt, Germany), at 70°C for 3 h.

The extract was filtered into a round-bottom flask using a vacuum pump (0.12 kW, 50/60 Hz, 230 + 10% V; ABM Greiffenberger Antriebstechnik GmbH, Marktredwitz, Germany) and filter paper (MN 615, 0.16 mm, weight 70 $g.m^{-2}$, average filtration capacity 4-12 µm,

filtration speed 22 s, ash content 0.1%, Macherey-Nagel & Co., Germany) and the ethanol was subsequently removed from the sample on a rotary evaporator at 30°C. The remaining extract was reconstituted with deionised water to a volume of 6.5 mL before being further filtered through a 0.45 µm hydrophilic PVDF syringe membrane filter (0.45 µm, 33 mm, Agela Technologies, Torrance, California, U.S) into 1.5 mL sample vials for HPLC analysis.

The LC Taste® separation was performed on a Hamilton Polymeric (PRP-1) column (250 × 10 mm, 10 µm) (Hamilton company, Nevada, U.S.) with an injection volume of 1 000 µL and a solvent flow rate of 3 $mL.min^{-1}$. The mobile phase consisted of (A) 0.1% formic acid in water ($v.v^{-1}$) and (B) ethanol, with a linear gradient from 0% to 100% B in 25 min.

The experiment was performed on an HPLC instrument, consisting of a HP 1050 Series (Hewlett-Packard, Palo Alto, California, U.S.) pump, autosampler and DAD (diode-array detector), set to collect chromatograms at 254 and 280 nm. The system was equipped with an Agilent 1100 Series degasser (Agilent Technologies, Waldbronn, Germany) and a Polaratherm column heater (Polaratherm, Series 9000, Sandra Selerity Technologies Inc., Salt Lake City Utah, U.S.) set to 80°C. Data was collected via ChemStation software (Agilent Technologies, Waldbronn, Germany).

The characteristic targeted, pungent fraction of the Pepperbark extract (Fraction 11) was collected between 10 and 11 minutes (3 mL) with a fraction collector (Model 2110, Bio-Rad, Hercules, California, U.S.).

Fraction 11, containing approximately 44% ethanol and 56% deionised water, was collected in duplicate, providing a fraction volume of 6 mL. The ethanol was evaporated partially under vacuum on a rotary evaporator at 30°C to a final volume of ~4 mL.

2.1.2 Extraction of volatile compounds

The remaining aqueous fraction (4 mL) was extracted with 2 mL DCM (dichloromethane) (≥99.8%, Sigma-Aldrich Merck, Darmstadt, Germany). The sample was vortexed for 2 min, followed by centrifugation for an additional 5 min to achieve separation between the organic (DCM) and the polar aqueous phase.

The bottom DCM layer was drawn out using a pipette and dried sodium sulfate salt before being concentrated using N_2 to ~80 µL. A volume of 1 µL of the DCM extract was injected into an Agilent 6890 series gas chromatography – mass spectrometry (GC-MS) system (Agilent Technologies, Palo Alto, CA, USA).

2.1.3 GC-MS Conditions

The GC-MS system was equipped with a split-splitless injector, operated in split mode in a ratio of 1:10 with the injector temperature set to 230°C. The column was a HP-FFAP (free fatty acid phase) of dimensions 30 m × 0.25 mm, 0.25 µm (Agilent technologies, Santa Clara,

United States). The carrier gas was helium with a constant flow rate of 1 mL.min^{-1}. The oven temperature started at 40°C, held for 5 min and ramped at a rate of 5°C.min^{-1} to a final temperature of 240°C, held for another 5 min. The gas chromatograph was coupled to a 5973 MS (Agilent Technologies, Palo Alto, CA, USA) with quadrupole and ion source temperatures set to 150°C and 230°C, respectively, and the filament emission current for electron ionisation (EI) used at the standard voltage of 70 eV.

Data analysis was performed using Chemstation software (Agilent, Santa Clara, United States) and compounds were tentatively identified by comparison of experimental mass spectra using Nist 02 (National Institute of Standards and Technology, Gaithersburg, MD, USA) and Wiley 275 (Wiley, New York, USA) spectral libraries.

2.1.4 Qualitative liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI-MS) and tandem mass spectrometry (MS/MS) analyses

LC-MS analyses were conducted on a Waters Acquity UPLC (ultra-performance liquid chromatography) instrument, equipped with a binary pump system, an in-line degasser, autosampler, column oven and DAD detector, set to collect UV-Vis (ultraviolet-visible) spectra in the range 230 to 500 nm (Waters, Milford, USA). The system was coupled to a Synapt G2 Q-TOF MS (quadrupole-time-of-flight; Waters) equipped with an electrospray ionisation (ESI) source, operated in positive ionisation mode. The pressure limit of 15 000 psi was used for the Waters BEH (ethylene bridged hybrid) column (C-18; 1.7 µm, 2.1 × 100 mm). A binary solvent system was used, consisting of a (A) water and (B) acetonitrile (Gradient-grade, Merck, Darmstadt, Germany), both containing 0.1% formic acid (v.v^{-1}). The solvent gradient started at 100% (A), held for 0.50 min, and decreased at a constant rate to 0% (A) in 12 min. The mobile phase flow rate was 0.4 mL.min^{-1} and the column temperature was set to 60°C. Fraction 11 was diluted in a 1:1 ratio with deionised water (1% formic acid; v.v^{-1}), and 2 µL was injected. MS mass calibration was performed using a sodium formate solution and leucine enkaphalin was used as the lock mass. The scan range was 120-1 500 amu for MS, and 100-1 500 amu for the MS/MS experiments, respectively. The capillary voltage was set to 2.5 kV and the sampling cone voltage to 15.0 V. The source and desolvation temperatures were 120°C and 275°C, respectively. The desolvation and cone gas flows (N_2) were 650 and 50 L.h^{-1}, respectively. For the MS data collection, a collision energy ramp of 20.0 V to 60.0 V was used. Data was processed using MassLynx version 4.1 software (Waters). Compounds were tentatively identified by comparison of retention indices, UV-Vis spectra and LC-MS spectra with literature. To confirm the identity of the major peak detected in the BPI chromatogram of the Pepperbark fraction, the relevant standard was purchased. A 1 mg sample of piperanine was purchased from Sigma-Aldrich (Modderfontein, Johannesburg, South Africa) and stored at -20°C. A stock solution was prepared by dissolving the sample in 500 µL of methanol

(≥99.9%, Sigma-Aldrich Merck, Darmstadt, Germany) in the original glass vial and sonicating the solution for 10 min at 20°C. The stock solution was stored at –20°C with N_2 in the vial headspace. A volume of 25 µL of the stock solution (2 000 ppm) was diluted with deionised water to a concentration of 200 ppm before being sonicated for 10 min at 20°C. This diluted solution was stored at –20°C with N_2 in the vial headspace for LC-MS analysis using the same conditions as specified above.

2.2 Quantitative analysis of piperanine in extracts of *Warburgia salutaris*

2.2.1 Experimental design

Using a solid to solvent ratio of 1:10 ($w.v^{-1}$), different solvents and extraction times were evaluated for the extraction of piperanine from *W. salutaris.*

The piperanine stock solution (2 000 ppm) was used to prepare a dilution series comprising concentration of 3.125 ppm, 6.25 ppm, 12.5 ppm, 25 ppm, 50 ppm, 100 ppm and 200 ppm, prepared through serial dilution with deionised water. Calibration was performed by LC-MS analysis of the piperanine dilution series, and the calibration curve obtained in this manner was used to quantify piperanine in the extracts.

2.2.2 Extraction conditions

A mass of 1 g of *Warburgia salutaris* (Pepperbark) dried leaf powder (Kerry Foods, Hillcrest, Durban, South Africa) was weighed and transferred into 20 mL glass vials with screw tops. A solid to solvent ratio of 1:10 ($w.v^{-1}$) was implemented, thus 10 mL of various solvents were measured out with a 10 mL pipette and added to the botanical leaf powder. Six extracts were created for each solvent combination: 100% ethanol, 50% aqueous ethanol ($v.v^{-1}$) and pH-adapted deionised water (pH 1.00, pH 4.00 and pH 7.00).

To create the pH-adapted solvents, phosphoric acid (85%; $w.v^{-1}$; Sigma-Aldrich, Merck, Darmstadt, Germany), was added dropwise to deionised water, while stirring the solution with a magnetic stir bar until pH 1.00 was achieved (pH-Meter, Crison, BASIC 20+, Spain). To create pH solutions 4.00 and 7.00, NaOH (2 M) (Sigma-Aldrich Merck, Darmstadt, Germany) was added dropwise to the divided pH 1.00 solution, constantly testing the pH while stirring the mixture with a magnetic stir bar until the desired pH's were obtained.

Three extracts prepared with each solvent were sonicated for 18 minutes at 50°C, while the remaining three extracts from each solvent was sonicated for 3 h at 50°C. After sonication the solids extracts were filtered into a round-bottom flask using a vacuum pump and filter paper (MN 615, 0.16 mm, weight 70 $g.m^{-2}$, average filtration capacity 4-12 µm, , filtration speed 22 s, ash content 0.1%, Macherey-Nagel & Co., Germany) into 10 mL sample vials with screw top lids. A 200 µL volume was pipetted from each of the extracts and filtered through a 0.45 µm hydrophilic PVDF syringe membrane filter into 1.5 mL sample vials. A

volume of 800 µL of deionised water was added to each sample vial to provide a 1:5 ($v.v^{-1}$) aqueous dilution of each extract. The diluted extracts were stored at 4°C, covered with foil, until LC-MS analysis (4 days).

3. RESULTS AND DISCUSSION

3.1 GC-MS RESULTS AND DISCUSSION

Figure 4.1 below shows the GC-MS chromatogram obtained for the analysis of the DCM extract of the pungent LC Taste® fraction. Table 4.1 lists the compounds numbered in Figure 4.1.

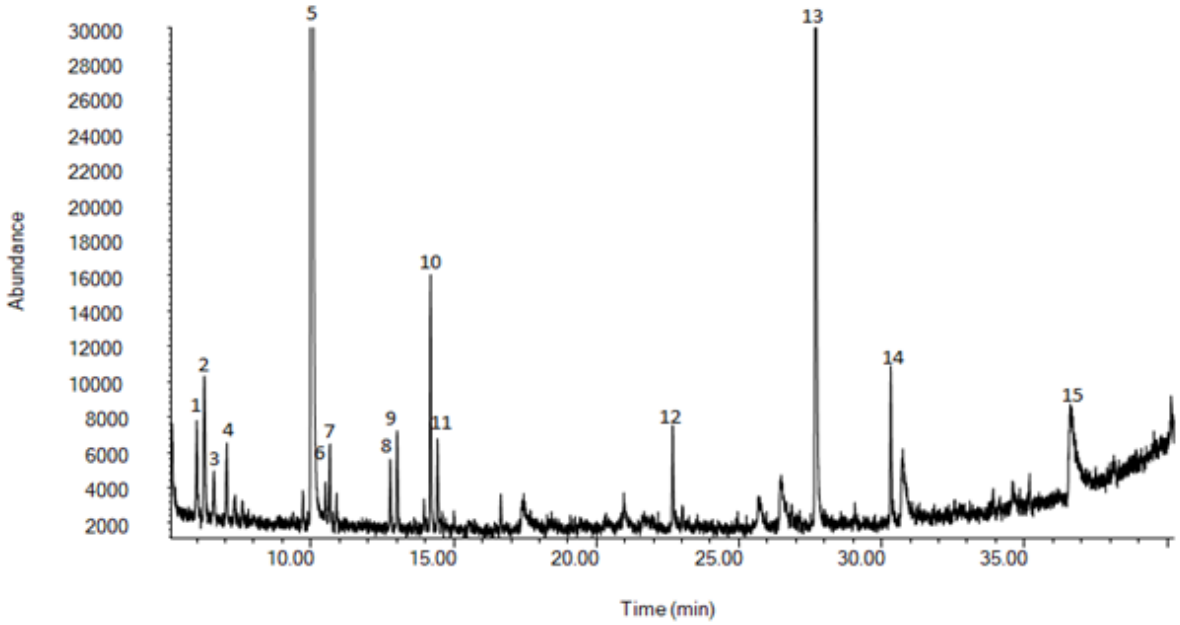

Figure 4.1 The GC-MS chromatogram of the DCM extract of the pungent LC Taste® fraction

Table 4.1 The analytes identified in the DCM extract of the LC Taste® pungent fraction with the numbers corresponding to the labelled peaks in Figure 4.1

Compound number	Retention time (min)	Compound name	Match quality	Compound properties
1	6.02	2-Hexanone	81	A ketone that is a natural constituent of foods, including milk, citrus fruits, nuts, bread and chicken (ATSDR, 1992).
2	6.29	1-Propanol	64	Naturally occurring alcohol found in various fruit and vegetables such as apples, honey, tea and pears (Christoph & Bauar-Christoff, 2007; Merck, 2020).
3	6.62	2-Pentanol	64	Volatile alcohol, present in multiple foods, including fruits, alcoholic beverages and cheeses (PubChem, 2020).
5	10.03	Isoamyl alcohol	90	Aliphatic alcohol with an aroma described as 'whiskey, fruity, banana' (Xu *et al*., 2019).
11	14.42	1-Hexanol	83	Associated with a typical green, grassy, and cucumber-like flavour (Christensen *et al.*, 2007; Xu *et al.*, 2019).
13	27.69	Benzene ethanol	95	Benzyl alcohol is an aromatic alcohol that occurs as a natural constituent of a number of plants, for example in some fruits and in green and black tea (EC, 2002).

Few volatile components were detected in the isolated fraction, which could be explained by the polarity (50% aqueous ethanol; $v.v^{-1}$) of the initial extraction conditions of the pepperbark leaf powder as well as the polarity of the targeted fraction, eluted in the middle region of the water-ethanol solvent gradient. The volatiles identified in the pepperbark fraction are common food volatiles, found in a number of fruit and vegetable products. None of the analytes detected via GC-MS could impart the characteristic peppery taste nor the pungency to the isolated fraction. However, the aromatic alcohol, 1-hexanol (compound 11) is a likely contributor to the underlying grassy flavour in the fraction (Christensen *et al.*, 2007; Xu *et al.*, 2019).

3.2 LC-ESI-MS AND LC-ESI-MS/MS RESULTS AND DISCUSSION

The base peak ion (BPI) chromatogram obtained for the LC-MS analysis of Fraction 11 of the Pepperbark extract is shown in Figure 4.2(A), below. The BPI chromatogram obtained for the analysis of the piperanine standard is shown in Figure 4.2(B). The retention times are indicated in black figures above the respective peaks, while their mass-to-charge (m/z) ratios are indicated in red. The compounds have been assigned numbers (1-12), indicated in bold black numbers above the respective peaks.

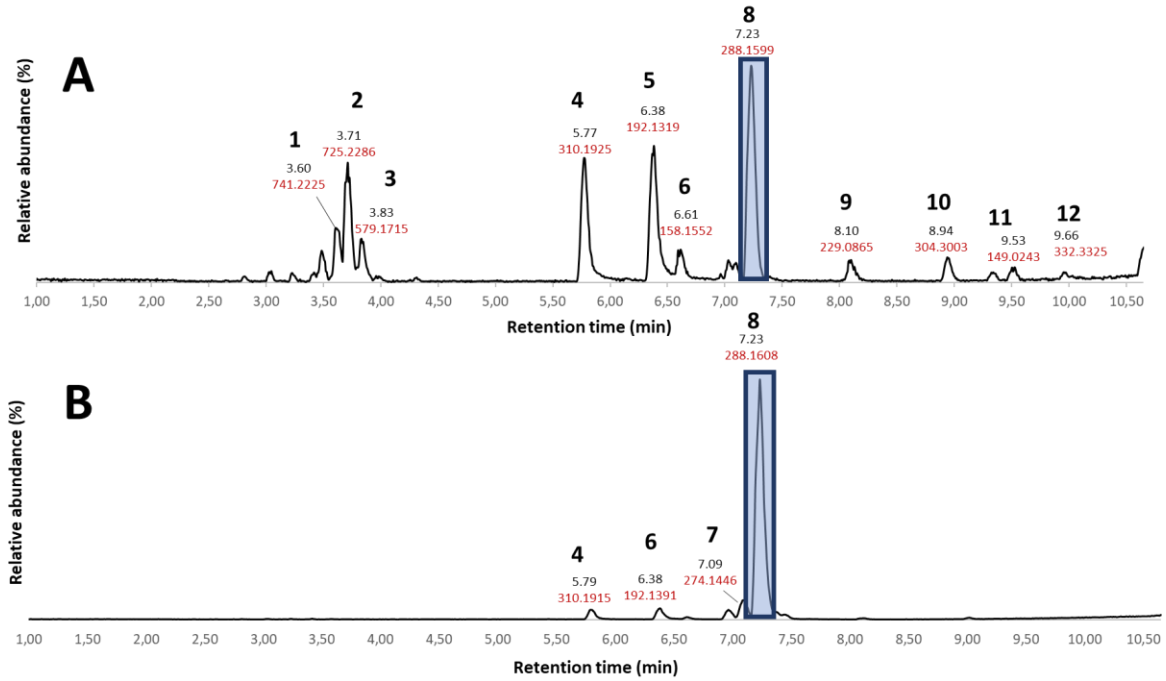

Figure 4.2 BPI chromatograms obtained for the LC-ESI-MS analysis of (A) the pepperbark extract Fraction 11, and (B) the analysis of a piperanine standard under the same conditions

The retention time (7.23 min) and the molecular ion mass (288 m/z, $[M + H]^+$) of the purchased standard of piperanine corresponds to the major peak in the BPI chromatogram of the Pepperbark fraction, as indicated by the blue-blocks enclosing the relevant peaks (Compound 8, Figure 4.2).

Comparing the high collision energy (MS^E) spectra of the most abundant peak in the pepperbark fraction BPI chromatogram (288 m/z) and the corresponding peak for the piperanine standard (Figure 4.3), it is evident that the accurate mass data and fragment ions match, confirming the identity of the main constituent of Fraction 11 as piperanine.

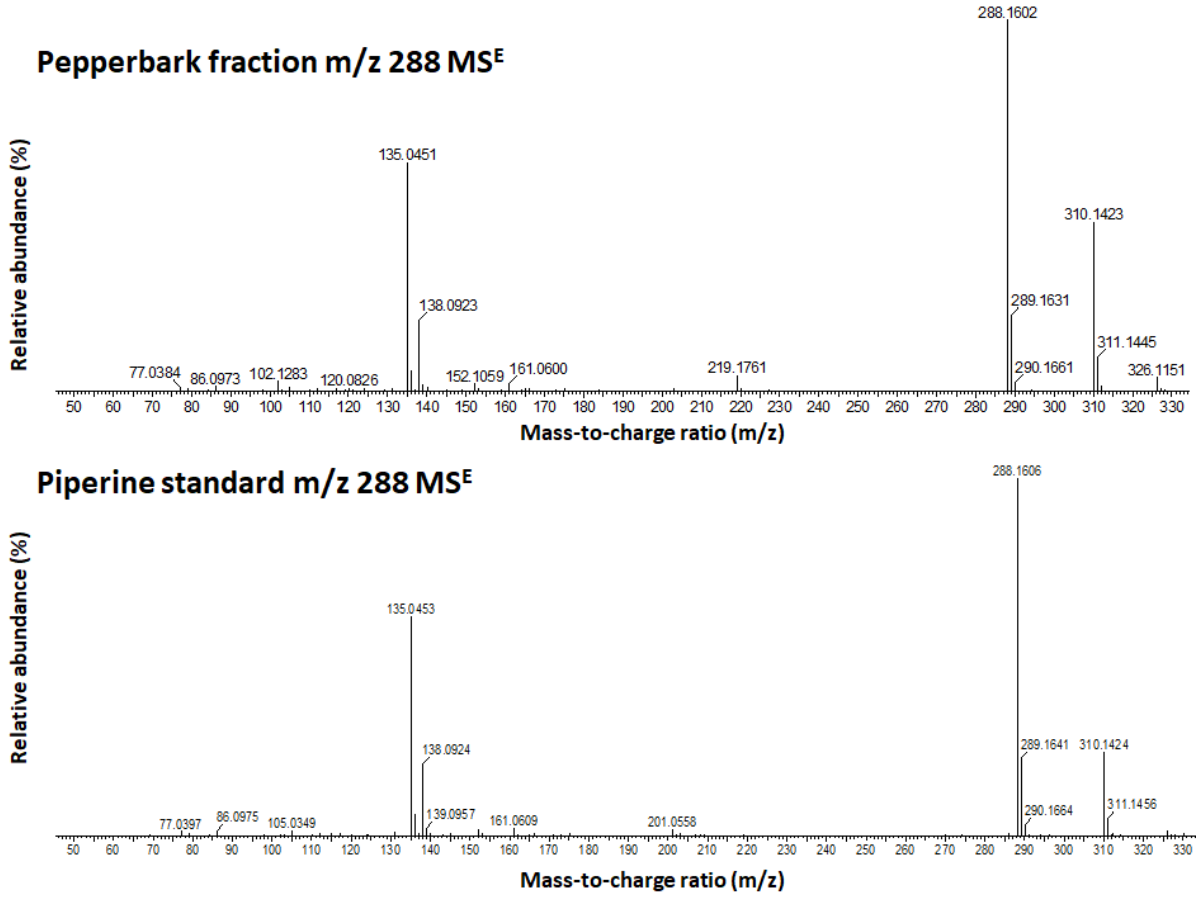

Figure 4.3 MS^E spectra obtained for the major chromatographic peak (Compound 8, m/z 288, indicated in blue) from the pepperbark Fraction 11 (above) and the piperanine standard (below)

The base peak at 288.1602 m/z corresponds to the molecular ion ($[M + H]^+$) of piperanine, $C_{17}H_{22}NO_3$ (Figure 4.4). The ion detected at 310 m/z corresponds to the sodium adduct of piperanine $[M+Na]^+$. The 135.0451 m/z fragment ion ($C_8H_7O_2^+$) is illustrated in Figure 4.5. The resulting methylenedioxybenzyl cation (135.0451 m/z) likely rearranges to form the stable methylenedioxytropylium cation (Figure 4.6), a well-known ion in MS (Addae-Mensa *et al.,* 1977). The ion at m/z 135 is commonly observed in the MS spectra of piperamides with a methylenedioxy-substituted aromatic ring bearing a benzylic methylene carbon, as in the case of piperanine (Liu *et al*., 2015; Wolff *et al.*, 2015).

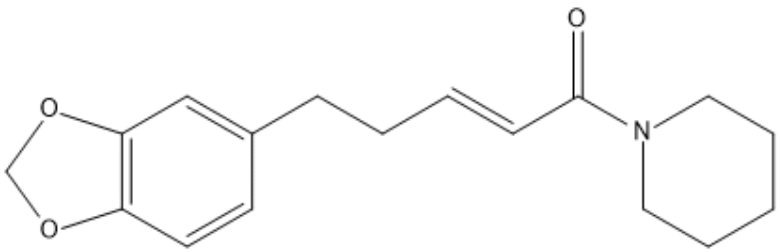

Figure 4.4 The molecular structure of the neutral species of piperanine ($C_{17}H_{21}NO_3$)

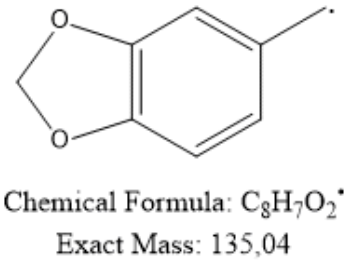

Figure 4.5 Piperanine fragment ion, methylenedioxybenzyl (m/z 135), detected in the MS^E spectrum of piperanine (Xu *et al.*, 2019)

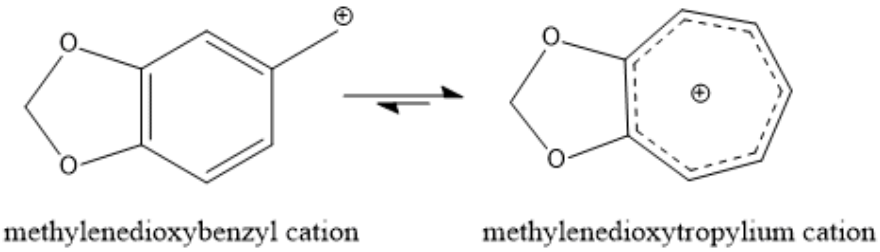

Figure 4.6 Methylenedioxybenzyl cation in equilibrium with the methylenetropylium cation, corresponding to the fragment ion peak at m/z 135 detected in the MS^E spectrum of piperanine

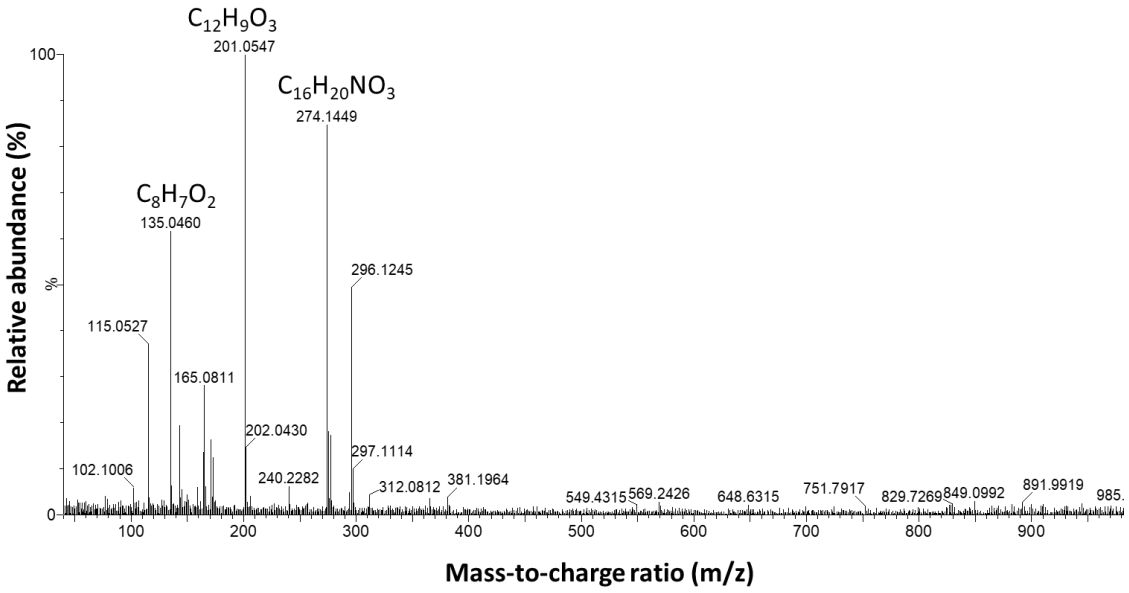

Figure 4.7 The MS^E spectrum of compound 7 in the piperanine standard (Figure 3B), with the molecular ion at m/z 274

Looking at the minor peaks present in the BPI chromatogram of the piperanine standard (Figure 4.2B), the fragmentation spectrum of peak 7 (m/z 274) is shown in Figure 4.7. This compound was tentatively identified as the pungent alkaloid, piperlonguminine, based on its molecular formula, $C_{16}H_{20}NO_3^+$ ($[M+H]^+$). Furthermore, the fragmentation behaviour observed for piperlonguminine (Figure 4.7) is in agreement with the product ions reported in the studies by Liu *et al*. (2011), as well as Xu *et al.,* in 2019. Both peak 4 and peak 5 were detected in the BPIs of both the piperanine standard and Fraction 11, with molecular ions at m/z 310 and m/z 192, respectively. These compounds could not be identified based on their MS^E spectra, but likely constitute degradation products of piperanine, since they are present in both the standard and the fraction of interest.

Several compounds detected at lower retention times in the pepperbark extract showed MS spectra indicative of flavonoids. The MS^E spectrum of the ion of peak 3, with a molecular ion detected at m/z 579 ($C_{27}H_{31}O_{14}^+$), is shown in Figure 4.8. The product ion detected at m/z 433, $C_{21}H_{21}O_{10}^+$, agrees with the formula of an apigenin flavone glycoside, likely either vitexin (apigenin-6-*C*-glucoside) or isovitexin (apigenin-8-*C*-glucoside) (Pereira *et al*., 2005). The wavelength of maximum UV absorbance (329 nm) confirms the molecule likely belongs to the flavone group of the phenolic compounds (Lin & Harnly, 2007). This product ion at m/z 433 is formed by a neutral of 146 amu from the molecular ion, indicative of the loss of an *O*-deoxyhexose unit, which is most likely attached to the glucoside group of vitexin/isovitexin (Pereira *et al*., 2005). The aglycone fragment ion was detected at m/z 313,

following the loss of 120 amu from product ion at m/z 433. This neutral loss is typical of the fragmentation of the glycosyl molecule of *C*-glycosylated species (Pereira *et al*., 2005).

The major phenolic peak (compound 2, Figure 4.2A), was characterised by a molecular ion at m/z 725 ($C_{33}H_{41}O_{18}^{+}$), and similar fragmentation behaviour as discussed for compound 3. The additional neutral loss of 146 amu suggests that peak 2 is the corresponding vitexin/isovitexin-di-*O*-deoxyhexoside.

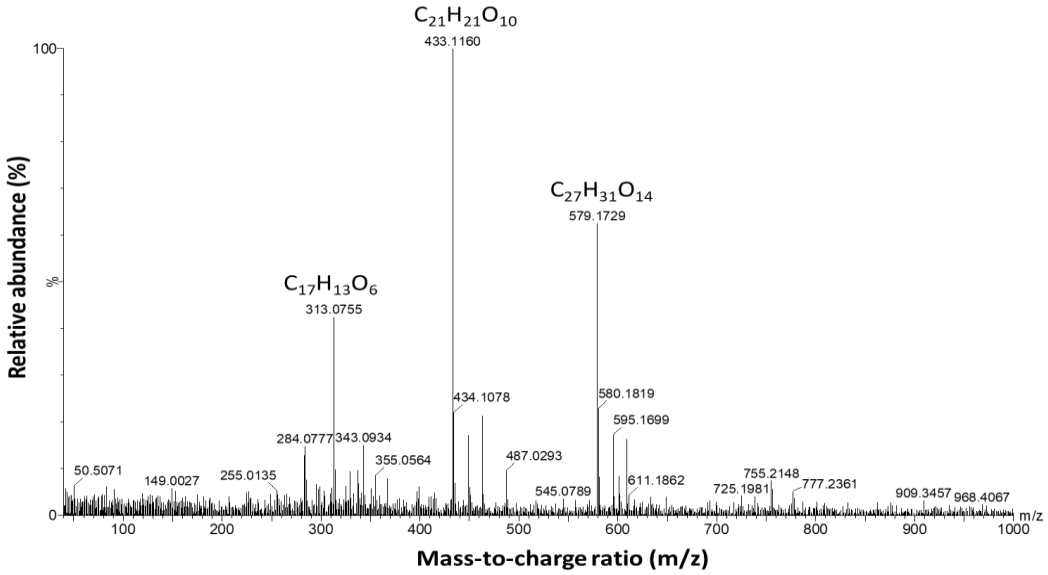

Figure 4.8 The MS^E spectrum of compound 3 in the pepperbark extract (Figure 3A), with the molecular ion at m/z 579

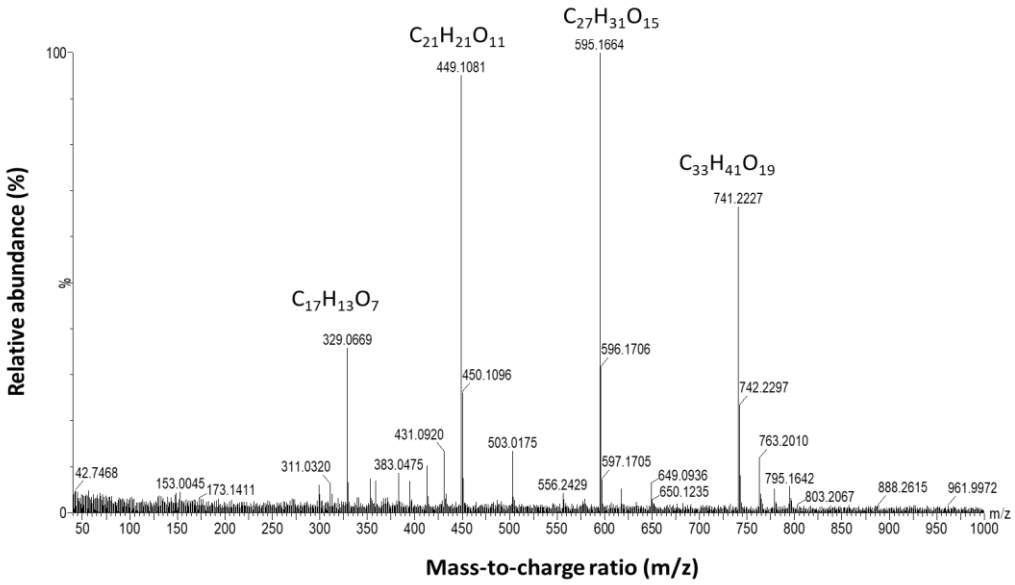

Figure 4.9 The MS^E spectrum of compound 1 in the pepperbark extract (Figure 3A), with the molecular ion at m/z 741

Compound 1, with the molecular ion at m/z 741 ($C_{33}H_{41}O_{19}^+$), showed a very similar fragmentation pattern (Figure 4.9) to compound 2. However, both the molecular ion and fragment ions are offset by 16 amu, with experimentally determined molecular formulae confirming the presence of an additional oxygen. Based on this information, compound 1 was tentatively identified as orientin (luteolin-6-*C*-glucoside), or isoorientin (luteolin-8-*C*-glucoside). The product ions at m/z 449 ($C_{21}H_{21}O_{11}^+$) and 595 ($C_{27}H_{31}O_{15}^+$) are thus formed by consecutive neutral losses of deoxyhexose groups, and corresponds to orientin (luteolin-6-*C*-glucoside) or its isomer, isoorientin (luteolin-8-*C*-glucoside) and orientin/isoorientin-*O*-deoxyhexose, respectively (Pereira *et al*., 2005).

Peak 6 (m/z 158) in the BPI chromatogram of the Pepperbark fraction (Figure 4.2A) showed no stable fragments under the MS^E conditions and could therefore not be identified. Its molecular formula, $C_9H_{20}NO^+$, suggests that it is a degradation product of piperanine.

Peaks 9 (m/z 229), 10 (m/z 304) and 12 (m/z 332) (Figure 4.2A) were all present in the blank analysis, and thus did not originate from the Pepperbark sample. Peak 11 (m/z 149) has the typical mass of a phthalate, a common laboratory contaminant (Figure 9) (Jeong *et al*., 2011).

Considering the compounds discovered in the Pepperbark fraction, the most abundant species detected in the isolated fraction, piperanine, was the compound identified as the sole contributor to the pungent taste of the fraction.

Piperanine, 4,5-dihydropiperine or $\Delta^{\alpha,\beta}$-dihydropiperine, is a piperine-type alkaloid (Navickiene *et al.,* 2000). Its structure is similar to that of piperine (Figure 4.10) (Navieckiene *et al.,* 2000).

Piperine is a naturally occurring, pungent alkaloid which principally contributes to the characteristic pungency of black pepper (*Piper nigrum*) (Wang *et al*., 2020). In addition to its immense value as a food spice, piperine has also been shown to possess diverse medicinal properties (Wang *et al*., 2020).

The bioactivities of active compounds from the *Piper* genus are very diverse, but can generally be categorised into two important areas: (1) as pesticides for agricultural application, and (2) as medicines to treat infections, tumours and inflammation, obesity and diabetes, depression and other human and animal physiological disorders (Chai & Elie, 2013; Mgbeahuruike et al., 2017).

In a study by Egebjerg *et al.* in 2009, it was shown that piperanine and piperine both have potential antiepileptic effects due to their affinity for the benzodiazepine site of the $GABA_A$ receptor. This effect on the central nervous system was attributed to the structural features of these compounds necessary for binding to the receptor, namely the length of the carbon chain, the presence of a conjugated double bond adjacent to the amide group, and the bulkiness of the amine group (Egebjerg *et al*., 2009). In 2008, Masuda *et al.* demonstrated the hepaprotective effect of piperanine, along with a number of amide-compounds, and explained the relevant structure-activity relationships. Furthermore, piperanine has been shown to enhance memory and is effective as an antimalarial compound, having antilarval properties (Mgbeahuruike *et al*., 2017; Piplani *et al.,* 2019). Muharini *et al.* (2015) demonstrated that piperanine possesses growth inhibition properties against the common plant pathogenic fungus, *Cladosporum cladosporiodes.*

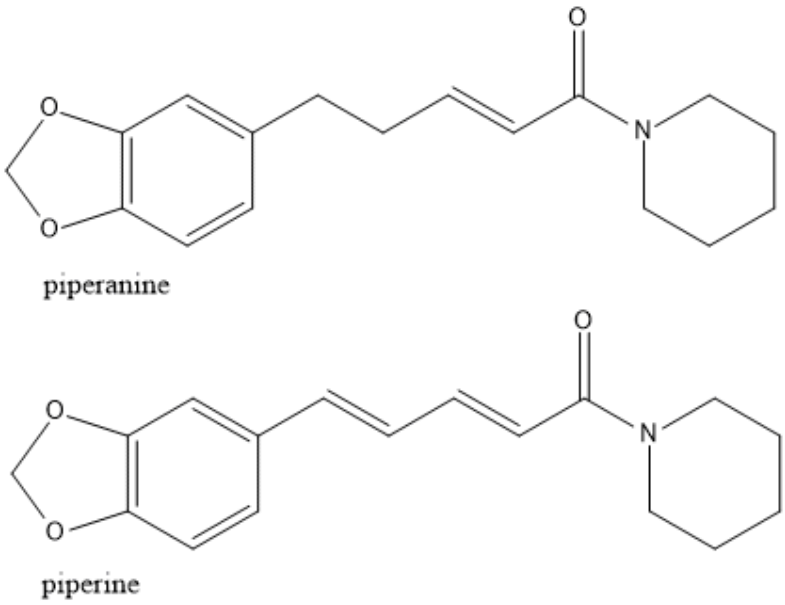

Figure 4.10 The molecular structure of piperanine (top) and piperine (bottom)

It was initially believed that the first known and most common constituent of the *Piper* species, piperine, was solely responsible for the pungency of black pepper (Traxler, 1971; Zachariah & Parthasarathy, 2008). In latter years, research revealed that other minor alkaloids also contributed, to a lesser extent, to the pungency of black pepper (Traxler, 1971; Govindarajan, 1922; Zachariah & Parthasarathy, 2008). In 1971, Traxler discovered piperanine as a contributor to the pungency of black pepper, using thin layer chromatography (TLC) coupled to organoleptic examination (Traxler, 1971). The identification of piperanine was based on infra-red (IR), ultraviolet (UV) and nuclear magnetic resonance (NMR) spectroscopic data and confirmed by the synthesis of the compound (Traxler, 1971).

The UV spectra indicated that piperanine holds close structural similarities to piperine but has a less conjugated system, as seen by comparing the figures presented in Figure 4.10 (Traxler, 1971; Govindarajan, 1977). The UV wavelengths at which maximum absorption occurred were at 233 nm and 283 nm, likely due to the absorption by two independent chromophores in the compound's structure (Traxler, 1971; Govindarajan, 1977).

Although piperanine has been detected in a variety of species belonging to the *Piper* genus of the *Piperaceae* family, including *Piper capense, P. longum, P. guineense, P. nigrum, P. rugosum*, *P. tuberculatum* and *P. chaba,* amongst others, the current study is the first report of piperanine in the species, *Warburgia salutaris,* as well as in the genus *Warburgia* (Navickiene *et al*., 2000; Matsuda *et al.*, 2009; Adewusi & Steenkamp, 2011; Mgbeahuruike *et al.*, 2017).

Because the chemical structures of piperanine, and piperine hold such a close resemblance (Figure 4.10), extraction conditions that result in high yields of piperine from pepper plants are likely to result in good yields of piperanine. Based on this assumption, piperanine was extracted from *Warburgia salutaris* leaf powder using parameters optimised for piperine extraction.

In an effort to intensify piperine extraction from the fruit of *Piper longum,* Rathod and Rathod (2014), showed that ultrasound-assisted extraction (UAE) resulted in far better yields than Soxhlet as well as batch solvent extraction. Through experimenting with various parameters, including solvent choice, extraction time, solid-to-solvent ratio and temperature of extraction, these authors reported that acetone was the best solvent, followed closely by ethanol and lastly, hexane (Rathod & Rathod, 2014). Because the yields of acetone and ethanol were very similar, ethanol was chosen as the preferred solvent as it is considered the safer, greener solvent with a lower cost and fewer handling concerns (Rathod & Rathod, 2014; Lin *et al.*, 2018). The optimal UAE conditions established, with ethanol as the extracting solvent, included an extraction time of 18 min, a solid to solvent ratio of 1:10 and a temperature of 50°C (Rathod & Rathod, 2014).

As discussed in the methods and materials section of this chapter, UAE was used in the present work with a constant solid-liquid ratio of 1:10 and the extraction temperature of 50°C. A range of different solvents were applied as well as two different extraction times (18 min and 3 h) in an attempt to maximise the quantity of piperanine extracted from the base plant material (Table 4.2).

Table 4.2 Extraction yields (parts per million) of piperanine extracted from *Warburgia salutaris* dried leaf powder using different solvents and extraction times

	18 minutes (ppm)	**3 hours (ppm)**
100% Ethanol	144	395
50% Ethanol	619	824
Water (pH 1.00)	127	35.8
Water (pH 4.00)	58.9	33.4
Water (pH 7.00)	40.8	70.4

Evidently, 50% aqueous ethanol extracted the maximum quantity of piperanine from the pepperbark leaves, with a more being extracted using the longer extraction time (3 h). For both ethanolic solvent systems, the longer extraction time enhanced the extraction of piperanine (Table 4.2). Concerning the pH-adapted aqueous solvent extractions, the pH 1 acidified water extraction yielded the best result, although lower extraction yields were obtained than those of the ethanolic solvent extractions. It is noteworthy that the piperanine yields of the acidified extractions (pH 1 and pH 4), decreased with increasing extraction time, and that the opposite was true for the neutral water extractions (Table 4.2).

Alkaloids typically occur in plants as salts of organic plant acids, while a few weaker basic alkaloids exist in inorganic salt or free base form (Rambo *et al.*, 2019). When extracted with inorganic acid acidified water, the organic acid of the alkaloid salt is replaced with the inorganic acid, increasing its solubility in water (Rambo *et al.*, 2019). As demonstrated in Table 3, the best aqueous extraction was at pH 1 (18 min), followed by pH 4 (18 min). At neutral pH, increasing the extraction time from 18 min to 3 h improved the extraction of piperanine (Table 4.2). While acidified water is a green solvent, suitable for food application, the extraction yields of piperanine were poor when compared to the extractions with the ethanolic solvent systems (Table 4.2) (Li *et al*., 2019). Since piperanine is a very weakly basic species, with a pKa of –0.79 ± 0.20, an extremely low pH would be required to fully protonate it, thus increasing its polarity, and so doing its solubility in water (Kotte *et al.,* 2014). The decrease in the concentration of piperanine observed for extended extraction times at pH 1 and pH 4 suggests that the molecule is susceptible to acidic hydrolysis (Table 4.2). Although no previous research

has investigated the stability of piperanine under stress conditions, the closely related compound, piperine, has been shown to undergo extensive degradation under both acidic and basic conditions (Kotte *et al.*, 2014). Furthermore, it has been shown that piperine is sensitive to oxidative stress, but is stable under neutral conditions (water), and withstands thermal as well and proteolytic stress (Kotte *et al.*, 2014). The results of this study suggest that the same might be true for the piperine analogue, piperanine – while the yields of the acidic extractions decrease with increasing extraction times, the piperanine extracted under neutral conditions increase with increasing time at elevated temperature (Table 4.2).

In the present study, the best extraction solvent system was 50% aqueous ethanol. This is in accordance with the polar nature of piperine-type amides, making polar organic solvents the suitable choice (Rambo *et al.*, 2019). An extension of the extraction time or/and an increase in temperature could possibly enhance the extraction yield, however, this was not evaluated.

The procedure below demonstrates the calculation of the mass of piperanine extracted per gram of *W. salutaris* leaf powder using 50% ($v.v^{-1}$) ethanol (3 h). The calculation assumes the density of the combination of ethanol and water (200 µL:800 µL) to be approximately equal to the density of water (assumed to be 1 000 $kg.m^{-3}$).

$$824\ ppm \approx 824\ mg.L^{-1}$$

$$C = m.V^{-1}$$

$$m = C.V = (824\ mg.L^{-1})(1\times10^{-3}\ L) = 824 \times 10^{-3}\ mg$$

$$C = Concentration;\ V = Volume;\ m = mass$$

Since the solution was diluted 5 times, the mass of piperanine extracted from 1 g *W. salutaris* dried leaf powder is 4.12 mg. In other words, approximately 4.12 mg of piperanine was extracted from 1 g of pepperbark dried leaf powder, constituting 0.412% ($w.w^{-1}$) of the sample.

Although this study is the first to discover and quantify the alkaloid piperanine in *Warburgia* species, the inherent natural variation of the biochemical composition of plant products is a crucial factor to consider (Chadare *et al.*, 2009; Bergh *et al.*, 2017). Genetic variation and growth conditions such as sun exposure, soil quality, water quality and quantity and fertiliser are all parameters that affects the phytochemical contents of botanical products (Chadare *et al.*, 2009; Bergh *et al.*, 2017). Additional batch-to-batch variations may be due to the origin of the sample (whether it is a mixture, from an individual tree, or obtained from a market), the age of the sample, treatment of the sample, the storage conditions, processing method and the analytical method applied (Bergh *et al.*, 2017). Only once multiple sources of

Warburgia salutaris leaf powder have been analysed for their piperanine content, can the piperanine content in this species accurately and reliably be estimated.

In order to put the quantity of piperanine found in *W. salutaris* leaves in the present work in context, knowledge regarding the pungency of piperanine is required. In 1977, Govindarajan composed a list of multiple naturally occurring pungent compounds, including piperine, its isomers and analogues, and related pungent compounds in ginger and capsicum. The pungency of the compounds, also referred to as heat or pain, was rated alongside their structures, as described by the Scoville heat unit (SHU) (Govindarajan, 1977). The pungency of piperanine is described as "half of that of piperine", as previously established by Traxler in 1971. The pungency of piperine is described by the rating of 200 000 SHU, thus the pungency of piperanine would be approximately 100 000 SHU (Traxler, 1971). The organoleptic evaluation of pungency, given in Scoville heat units, is based on a dilution procedure, expressing the result as the reciprocal of the threshold or lowest concentration at which pungency is detected by a panel (Traxler, 1971). Although the test is theoretically simple, in practice the reproducibility is poor due to multiple sources of variation (Govindarajan, 1977). Although Traxler (1971) stated that the Scoville method was followed as described in U.S. Federal Spice Specifications of 1962, his rating of the pungency of piperanine is the only available result, raising concerns about its reliability.

While the amount of piperine varies between the *Piperaceae* species, values of between 2% and 7.4% ($w.w^{-1}$) have been reported in both black and white pepper (*P. nigrum*) (Gorgani *et al.*, 2016). Comparing these percentages of piperine in pepper in conjugation with the SHU of piperine (200 000 SHU) with the percentage of piperanine (0.412%, $w.w^{-1}$) in *W. salutaris* dried leaf powder (100 000 SHU), Pepperbark leaf powder certainly does not offer a more economic source of pungency than *P. nigrum* fruit for culinary purposes.

Although piperanine was likely the major source of pungency detected in the peppery fraction isolated from the *W. salutaris* extract, it has been reported that sesquiterpenes may also contribute to the pungency detected in *Warburgia* species (Leonard & Viljoen, 2015; Szallasi *et al.,* 1998).

The pungent sesquiterpenes, warburganal, polygodial, mukaadial, isopolygadial, salutarisolide, have previously been isolated from the stem bark of a pepperbark tree from the Soutspanberg in Limpopo province (Mashimbye, 1999), while muzigadial was isolated from *W. salutaris* by Rabe and van Staden for the first time in 2000.

The most abundant drimane sesquiterpene in *W. salutaris* bark was found to be ugandensidial at 0.23% ($w.w^{-1}$), while muzigadial (0.01%), polygodial (0.03%) and warburganal (0.06%) were present in lower concentrations (Drewes *et al*., 2001). The two major bioactive compounds, polygodial and warbuganal, were quantified in leaf extracts of the same Zululand genotype, to find the concentrations of 0.06% ($w.w^{-1}$) and 0.05%, respectively

(Drewes *et al.*, 2001). The figures presented in the study by Drewes *et al.* (2001) represent the isolated quantities of the sesquiterpenes, and their concentrations are therefore expected to be far greater in the plant material, since the laborious separation techniques result in inevitable loss of the targeted analytes.

Research has shown that these drimane sesquiterpene derivatives contribute to the remarkable biological activity of the *Warburgia* species (Rabe & Van Staden, 2000; Leonard & Viljoen, 2015; Khumalo *et al.*, 2019). They have been shown to possess antimycobacterial, antibacterial, antifeedant, antifungal, anti-inflammatory as well as antimycotoxigenic activity (Rabe & Van Staden, 2000; Leonard & Viljoen, 2015; Khumalo *et al.*, 2019). Warburganal, polygodial and muzikadial have also been reported to possess strong cytotoxicity against cancer cell lines (Khumalo *et al.*, 2019). A significant finding relating to the structure–activity relationship of these compounds suggests that the enal-9β-aldehyde moiety is essential to biological activity (Figure 4.11) (Rabe & Van Staden, 2000).

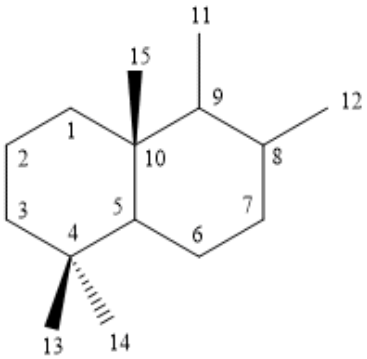

Figure 4.11 Drimane sesquiterpene structural numbering

In a study of the pungency of the coriander plant, Laksa (*Persicaria odorate)*, commonly used in Thai food, the responsible compound was discovered to be polygodial (Cayeux & Starkenmann, 2017). The pungency was said to be different from the pungency or tingling sensation known for piperanine, capsaicin, gingerol and spilanthol (Cayeux & Starkenmann, 2017). A sensory study of polygodial, piperine and capsaicin, performed by a panel of 25 individuals, indicated that polygodial is twice as strong as piperine and 40 times weaker than capsaicin (Cayeux & Starkenmann, 2017). Another source describes the taste of polygodial as sharp and pungent, similar to black pepper, however, without any accompanying flavour (Ravindran, 2017).

The use of polygodial or polygodial-containing plant extracts has been patented (United States Patent no. 5523105) to enhance mint flavour in food and beverages, cosmetics and pharmaceuticals (Leonard & Viljoen, 2015; Mortzel & Le Bon, 2016). Addition of polygodial improves the taste of the product by enhancing the cooling sensation as well as

increasing the lingering sensation of the mint flavour and odour (Leonard & Viljoen, 2015; Mortzel & Le Bon, 2016). This property is particularly valuable in the manufacturing of chewing gum and toothpaste (Leonard & Viljoen, 2015). Polygodial, isolated from *W. stuhlmannii*, has successfully been applied to sweetened beverages to enhance sweetness, reduce after-taste and improve flavour (US Patent no. 5948460) (Leonard & Viljoen, 2015; Mortzel & Le Bon, 2016). Concentrations between 10 and 1 000 ppb enhanced sweetness, while reducing aftertaste (Leonard & Viljoen, 2015).

Considering the content of sesquiterpenoid unsaturated dialdehydes of *W. salutaris* leaves, the potential for flavour applications in the food and beverage industry could be far greater when these pungent metabolites are extracted, together with the pungent alkaloid, piperanine.

Sesquiterpenes are C15-terpenoids, composed of three isoprene units (Awouafack *et al.*, 2013). Primary sesquiterpenes are linear or cyclic hydrocarbons, without substituents, making them very non-polar (Jiang *et al.*, 2016). While terpenes with 15 carbons or less are generally volatile due to their small size and low polarity, these terpene scaffolds are often modified by hydroxylation, glycosylation, acylation, and aroylation, which alters the physical size and nature of the terpene molecule, and can increase their polarity and decrease their volatility (Figure 4.12) (Jiang *et al.*, 2016).

The major bioactive, pungent sesquiterpenes associated with *W. salutaris* are oxidation products of the drimane sesquiterpenoid skeleton, rendering compounds that are less volatile and slightly more polar than their hydrocarbon counterparts (Figure 4.12) (Rabe & Van Staden, 2000).

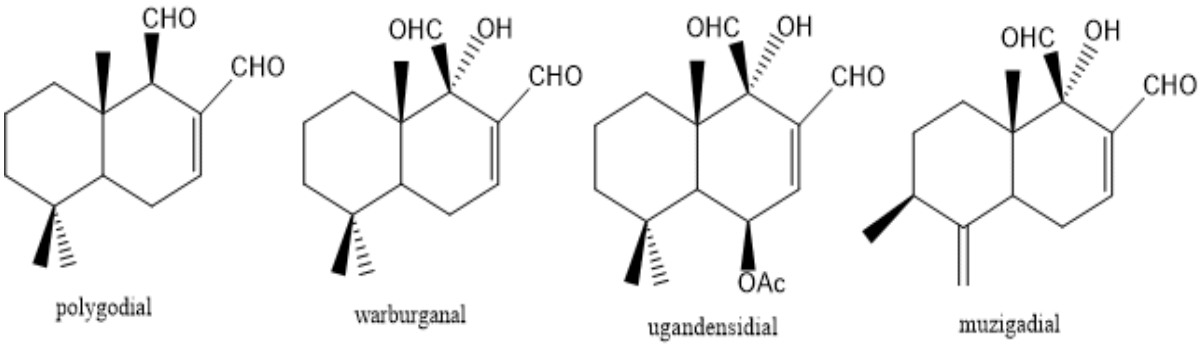

Figure 4.12 Molecular structures of pungent sesquiterpenoid dialdehydes from *W. salutaris* bark

The drimane sesquiterpenes, polygodial and warburganal, known to be present in the leaves of *Warburgia salutaris*, were not extracted in the pungent fraction isolated via LC Taste®. In previous studies, sesquiterpenes have been extracted from the bark and leaves of *Warburgia* species using the non-polar solvents, *n*-hexane, DCM, as well as the moderately

polar solvents such as ethyl acetate and a combination (1:1; $v.v^{-1}$) of methanol:DCM (Kubo, 1995; Rabe & Van Staden, 2000; Drewes *et al.,* 2001; Khumalo *et al*., 2019). The extraction times were typically from 8 h, or longer, at temperatures lower than 40°C, using either maceration or Soxhlet extraction (Kubo, 1995; Rabe & Van Staden, 2000; Drewes *et al.,* 2001; Khumalo *et al*., 2019). In most of these studies, the sesquiterpenes were separated using column chromatography, on a silica gel column, using mobile phase gradients of hexane and ethyl acetate (Rabe & Van Staden, 2000; Drewes *et al.,* 2001; Khumalo *et al*., 2019). Kubo (1995) reported that the use of methanol or other alcoholic solvents either partially or totally inactivated antifungal properties of these drimane sesquiterpenes acetal or hemiacetal formation.

It is therefore suspected that the initial extraction conditions of 50% aqueous ethanol ($v.v^{-1}$) excluded the extraction of the less polar *W. salutaris* drimane sesquiterpenes. In addition, the extraction solvent, ethanol, has a possible inhibitory effect on the bioactivity of the sesquiterpenes (Kubo, 1995).

In essence, preparations of *Warburgia salutaris* leaves, targeted at extracting maximum peppery, pungent components, drimane sesquiterpenoid dialdehydes as well as the piperine-type alkaloid, piperanine, potentially hold value as a novel flavour source in food and beverage products.

Due to the numerous medicinal properties associated with piperanine as well as the sesquiterpenoids, the extracts can therefore offer a valuable ingredient to be used in functional beverages (Rabe & Van Staden, 2000; Egebjerg *et al.*, 2009; Leonard & Viljoen, 2015; Mgbeahuruike *et al.,* 2017; Khumalo *et al.*, 2019; Piplani *et al*., 2019).

The functional food and beverage market represents one of the biggest and most rapidly growing food markets. This is due to increasing consumer awareness regarding health and wellness, which drives consumers towards healthy diets, preventive care, and secondary source medication (Nazir *et al.*, 2019). Coupled to the exponential growth in the functional food market, there is huge potential for the development of functional beverages (Nazir *et al.*, 2019). If the approach of a functional food product is followed, the science-based demonstration of the health benefits is required for the validation of health claims and successful marketing (Nazir *et al.*, 2019).

Even if concentrations of bioactive components are too low to make any health-claims, through proper marketing, consumers will be attracted to the natural flavour source and its associated benefits (Gruenwald, 2009; Harnly *et al.,* 2017; Nazir *et al*., 2019).

4. CONCLUSION AND RECOMMENDATIONS

In this research chapter, the pungent, peppery flavour fraction collected from the *Warburgia salutaris* leaf extract via LC Taste® was analysed by GC-MS and LC-ESI-MS to identify and quantify the compounds responsible for the characteristic taste of the fraction.

The GC-MS results suggested that very few aromatic compounds were present in the flavour fraction collected. The limited extraction of volatile components can be explained by the high polarity of the initial extraction solvent system (50% aqueous ethanol; $v.v^{-1}$) used to extract the leaf powder. Similarly, the collected LC Taste® flavour fraction of interest eluted in the middle region of the water-ethanol gradient, suggesting that relatively polar analytes were collected.

The results of the LC-ESI-MS analysis of the pungent Pepperbark fraction enabled the identification of the most abundant compound in the fraction, namely the pungent piperine-type alkaloid, piperanine. This piperine analogue is thought to be the major contributor to the pungency detected in the isolated fraction. Although piperanine has been reported in a variety of species belonging to the *Piper* genus of the *Piperaceae* family, the current study is the first to report piperanine in the species, *Warburgia salutaris,* and in the genus *Warburgia.*

The extraction conditions, 50% aqueous ethanol ($v.v^{-1}$), and an extraction time of 3 h, proved to be the most efficient in the extraction of piperanine from the dried leaf powder of *W. salutaris.* The extraction technique, UAE, extraction temperature (50°C), and solid-solvent ratio (1:10) was kept constant and were selected based on the parameters that extract maximum piperine from long pepper (*P. longum*).

Since piperine-type amides are polar in nature, polar organic solvents are effective extraction solvents. While the best extraction of piperanine was yielded in the 50% aqueous ethanol at 3 h, perhaps an extension of extraction time or increased extraction temperature could further enhance the extraction.

Although no previous research has investigated the stability of piperanine under stress conditions, the extraction results suggest that piperanine is susceptible to acidic hydrolysis and is stable at neutral pH and under mild thermal stress. This result agrees with the stability of the closely related compound, piperine, that has been shown to undergo extensive degradation under acidic conditions but is stable under the same conditions as piperanine.

Considering the most effective extraction conditions, approximately 4.12 mg of piperanine was extracted from 1 g of pepperbark dried leaf powder, constituting 0.412% ($w.w^{-1}$) of the sample. This quantity, as with any phytochemical of natural origin, is expected to be subject to variation and therefore accurate and reliable calculations can only be made once multiple sources of *Warburgia salutaris* leaf powder have been analysed for their piperanine content.

The pungency of piperanine has been estimated in previous work as half of the pungency of piperine. Considering this, as well as the respective contents, with the discovered piperanine content of *W. salutaris* dried leaf powder (0.412%, $w.w^{-1}$) and the piperine content in *Piperaceae* species ranging from 2% to 7.4% ($w.w^{-1}$), Pepperbark leaf powder certainly does not offer a more economic source of pungency than *P. nigrum* fruit for culinary purposes.

Although the pungency detected in the fraction isolated from the *W. salutaris* extract was ascribed to piperanine in the current study, other hot-tasting compounds, classified as sesquiterpenes, have been reported in *Warburgia* species, and are known to contribute to the heat associated with the plants belonging to this genus.

The drimane sesquiterpenes, polygodial and warburganal, have been reported in the leaves of *Warburgia salutaris*, but were not extracted in the pungent fraction, isolated via LC Taste®. Non-polar or moderately polar solvents, in combination with long extraction times and moderately low extraction temperatures were used in previous studies in which sesquiterpenes were successfully extracted from *Warburgia* spp. leaves and bark. The polar extraction conditions of 50% aqueous ethanol ($v.v^{-1}$) used in this study thus excluded the extraction of the non-polar drimane sesquiterpenes.

If these pungent metabolites could be extracted together with the pungent alkaloid, piperanine, the potential for flavour applications in the food and beverage industry could be far greater.

Due to the dramatically growing market for functional foods and food and beverage products with natural flavours, colours and preservatives, extracts of *Warburgia salutaris* leaves with enhanced extraction yields of piperanine as well as pungent sesquiterpenoids, potentially hold great value, with the attractive features including the novelty of the flavour and the numerous medicinal properties associated with piperanine and the sesquiterpenoids.

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CHAPTER 5

GENERAL DISCUSSION, RECOMMENDATIONS AND CONCLUSIONS

For successful LC Taste® fractionation, concentrated extracts were prepared from each of the botanical matrices, *Adansonia digitata* (Baobab), *Cyclopia genistoides* (Honeybush), *Moringa oleifera* (Moringa) and *Warburgia salutaris* (Pepperbark) by manipulating multiple extraction parameters, namely the concentration of ethanol in the water-ethanol solvent, the extraction time at an elevated temperature (70°C), the solid-liquid ratio as well as the extraction technique, comparing the extraction efficiency of sonication (UAE) to maceration with stirring.

A hydroalcoholic solvent system of aqueous ethanol proved to be a suitable extraction solvent that is essentially safe for subsequent sensory evaluation (Dirar *et al.*, 2019; Mustafa & Turner, 2019). The solvent choice, 50% ethanol concentration ($v.v^{-1}$) extracted the optimum taste and flavour from each botanical, a decision guided by sensory evaluation of the resulting extracts (Dirar *et al.*, 2019; Mustafa & Turner, 2019). This was the expected outcome since many researchers have previously reported 50% ethanol ($v.v^{-1}$) as an effective solvent for the extraction of phytochemicals from plant-based matrices (Durling *et al*., 2007; Azmir *et al.,* 2013; Chiang *et al.,* 2017; Bamba *et al.,* 2018; Anbalagan *et al.,* 2019; Mustafa & Turner, 2019).

In the present study, the solids concentration of each botanical was maximised, producing concentrated extracts. It is, however, likely that an excessive solid-liquid ratio was used for the botanicals, resulting in solute saturation (Zhang *et al.,* 2019). Since the solid-liquid ratio is an important factor that affects the extraction efficiency, it is recommended that in future research, a wider range of smaller ratios of solid-liquid should be applied to enhance the extraction efficiency of the extractions.

When comparing the extraction techniques in this study, maceration with magnetic stirring resulted in better extraction of flavour from Honeybush, Moringa and Baobab, while sonication resulted in better flavour extraction from Pepperbark. Both techniques offer unique benefits and are suited for extraction of phytochemicals from botanical sources (Vintatoru, 2001; Rostagno *et al.*, 2003; Azmir *et al*., 2013; José & Pérez, 2019). Different extraction methods offer alternatives to meet different objectives with different desired outcomes (Vintatoru, 2001; Rostagno *et al.*, 2003; Azmir *et al*., 2013; José & Pérez, 2019). Suitable alternative extraction techniques that could be applied to herbal products are microwave-assisted extractions, supercritical fluid extraction, enzyme-assisted extractons, pressurised liquid extractions, and electric field-assisted extractions (Mandel & Tandey, 2016; Belwal *et al.*, 2018). These methods of extraction are rapid, simple, environmentally friendly, fully automated and typically render high quality final extracts that are rich in the targeted compounds (Belwal *et al*., 2018).

The effect of the extraction time at elevated temperature (70°C) on the taste and peak intensities in the generated DAD (diode-array detection) chromatograms indicated that 24 h extracted the maximum flavour from Honeybush. This outcome suggests that the compounds responsible for flavour in Honeybush tea can withstand thermal degradation to a certain degree.

In contrast, the taste- and peak intensities of Pepperbark, and Moringa, declined as a function of time at elevated temperature (70°C). Three hours and 6 h resulted in similar taste and peak intensities for Baobab, however 24 h stirring resulted in the least efficient extraction. This outcome suggests that the compounds responsible for the taste of these botanical samples are not stable to thermal stress. In future work, it would be valuable to observe the effect of a wider temperature range, using both UAE (ultrasound-assisted extraction) and maceration with magnetic stirring on the flavour extracted from various botanical sources.

Although the extraction parameters could be further optimised for each of the botanicals, for the purpose of this study, the selected extraction parameters produced suitably concentrated extracts for flavour screening. Additional factors that would improve the repeatability of the results would be to use multiple samples of each of the botanicals, considering the natural variation of botanical products. Similarly, a larger preliminary and expert sensory panel would improve the reliability of the study outcomes.

LC Taste® was successfully applied to each of the botanical extracts, each with a unique water-ethanol solvent gradient and fraction-collection period, guided by the occurrence of DAD signals. The fractionation of each of the botanicals enabled screening for flavours and tastes unique to each extract, without dominant tastes typically associated with botanicals, for example bitterness or sourness, from adjacent fractions overpowering underlying flavours or tastes.

A large injection volume, high mobile phase flow rate and a short analysis time allowed for sufficient fraction volumes with detectable flavour to be collected for sensory evaluation in a timely manner. Although the selected parameters resulted in relatively poorly resolved chromatograms, the aim of LC Taste®, to rapidly screen complex extracts for potentially valuable flavours/tastes, was successfully achieved (Reichelt *et al*., 2010a, Reichelt *et al*., 2010b; Reichelt *et al*., 2010c).

To obtain better resolved chromatograms, the injection volume can be reduced, although this would reduce the flavour intensity of the eluted flavour fractions. For this reason, concentrated botanical extracts are essential, to maximise the flavour in the sample to be injected. Similarly, the mobile phase flow rate could be reduced, and therefore smaller fraction volumes would be collected consequently, thus increasing the analysis time, especially if multiple runs would be required to collect sufficient fraction volumes. The operating parameters should be balanced when applying LC Taste®, as they were in this study, to

achieve acceptable separation efficiency as well as sufficient fraction volumes with maximum flavour.

The *Adansonia digitata* fractions were generally sour in taste, with varying intensities. In the case of *Moringa oleifera, Warburgia salutaris* and *Cyclopia genistoides*, the fractions that eluted in the middle region of the gradient, held the most flavour potential. This outcome correlates with the previously reported result that the 50% ethanol ($v.v^{-1}$) extracted the preferred flavour profile from each of the botanical extracts initially. At higher concentrations of ethanol, no tastes were detected, due to the overwhelming taste of the alcohol.

The aim of this study, to explore the discussed botanicals for potentially valuable flavours and tastes, was thus successfully achieved by applying LC Taste® as a flavour screening method. The study enabled the evaluation of the technique itself, LC Taste®, which proved to be an exceptionally useful flavour screening technique that enables the rapid screening of complex extracts for non-volatile constituents. The collected flavour fractions could be directly tasted by a sensory panel, without the need to remove harmful solvents, thereby protecting the compounds in the fraction from chemical alteration and/or destruction. The method is well described by Reichelt *et al.* (2014) as an HTLC-coupled, guided sensory analysis, with the occurrence of DAD peaks guiding the fraction collection and tasting procedure. The outcome of this study confirms that LC Taste® is a valuable flavour screening method, providing an accelerated technique to identify potentially valuable flavours from complex extracts (Reichelt *et al.* 2010a; Reichelt *et al.,* 2010b, Reichelt *et al*., 2010c, Mittermeier *et al.,* 2018).

The microbial safety of botanical preparations is an important safety parameter to consider, as with any other food ingredient (Trucksess & Scott, 2008; Thanh *et al*., 2018). The microbial results of this study indicated that the botanical fractions were all free from the pathogenic microbes, *Salmonella* spp. as well as *Bacillus cereus* spores, suggesting that the obtained fractions are microbiologically safe for human tasting.

The expert sensory panel were particularly interested in a pungent, peppery fraction (Fraction 11) collected from the *Warburgia salutaris* dried leaf powder, described as having a pungent taste and a distinctive, lasting, tingling sensation in the mouth (Dovey, M. 2019, Technical Manager, Kerry Ingredients and Flavours, Durban, South Africa, personal communication, 17 November). This particular fraction was therefore accepted for further analysis to identify the specific compounds responsible for the perceived sensory results. Although LC Taste® is a powerful screening tool, it is not intended to replace conventional characterisation and evaluation of interesting single compounds (Reichelt *et al*., 2010b; Reichelt *et al*., 2012).

The flavour fraction collected from Pepperbark via LC Taste®, was analysed via gas chromatography-mass spectrometry (GC-MS) as well as liquid chromatography-electrospray

ionisation-mass spectrometry (LC-ESI-MS) and tandem mass spectrometry (MS/MS) to identify and quantify the compound(s) responsible for the characteristic taste of the fraction.

The GC-MS results suggested that very few aromatic compounds were present in the flavour fraction collected. The proposed reason for the limited extraction of volatile components is the high polarity of the initial extraction solvent system (50% aqueous ethanol; $v.v^{-1}$) used to extract the leaf powder. In addition, the collected LC Taste® flavour fraction of interest eluted in the middle region of the water-ethanol gradient, suggesting that relatively polar analytes were eluted.

The results of the LC-ESI-MS and MS/MS analysis of the pungent Pepperbark fraction enabled the identification of the major constituent of the fraction, namely the piperine-type alkaloid, piperanine. Piperanine is thought to be the compound responsible for the pungency detected in the isolated fraction. The current study is the first to report the presence of piperanine in *Warbargia salutaris*, as well as in the genus, *Warburgia*.

Based on the parameters that extracted the maximum piperine yield from the fruit of *P. longum* (Rathod & Rathod, 2014), UAE was selected as the extraction technique to extract piperanine from the dried leaf powder of *W. salutaris*. The extraction temperature (50°C) and the solid-solvent ratio (1:10) were selected in the same way. The solvent, 50% aqueous ethanol ($v.v^{-1}$) and extraction time of 3 h, were found to be the most efficient in the extraction of piperanine based on the results of the present study.

Although no previous studies have investigated the resistance of piperanine to stress conditions, the extraction results indicate that piperanine is susceptible to acidic hydrolysis but can withstand neutral pH as well as mild thermal stress. This result is in accordance with the stability of the closely related compound, piperine, that has been reported to degrade under acidic conditions, but is stable to neutral pH conditions and thermal stress (Kotte *et al.*, 2014). Considering these results, an extension of extraction time or increased extraction temperature could potentially enhance the extraction yield of piperanine.

The most efficient extraction conditions resulted in the extraction of approximately 4.12 mg of piperanine from 1 g of pepperbark dried leaf powder, constituting 0.412% ($w.w^{-1}$) of the sample. As with any phytochemical of natural origin, this quantity is subject to variation. Ideally, multiple sources of *Warburgia salutaris* leaf powder should be analysed for their piperanine content before reliable and accurate calculations of this constituent can be made (Chadare *et al*., 2009; Bergh *et al.*, 2017).

The pungency of piperanine has been reported as “half of that of piperine” (Traxler, 1971; Govindarajan, 1977). In Scoville Heat Units (SHU), the pungency of piperine is rated as 200 000 SHU, thus the pungency of piperanine would be approximately 100 000 SHU (Govindarajan, 1977).

The piperine concentration of the *Piperaceae* genus varies among the species, with reported values of between 2% and 7.4% ($w.w^{-1}$) in both black and white pepper (*P. nigrum*) (Gorgani *et al.*, 2016). By comparing the piperine content of pepper together with the SHU of piperine (200 000 SHU) to the discovered piperanine (0.412%) content of *W. salutaris* dried leaf powder (100 000 SHU), it is clear that Pepperbark leaf powder does not offer a more economic source of pungency than *P. nigrum* fruit for culinary purposes.

Although piperanine was responsible for the pungency detected in the peppery fraction isolated from the *W. salutaris* extract, other hot-tasting compounds, classified as sesquiterpenes, have been associated with the *Warburgia* species, and contribute to the heat associated with these plants (Leonard & Viljoen, 2015; Szallasi *et al.,* 1998).

The pungent metabolites, polygodial and warburganal, known to be present in the leaves of *Warburgia salutaris*, were not extracted in the pungent fraction isolated by LC Taste® (Drewes *et al*., 2001). In previous studies, these sesquiterpenes have been extracted from the bark and leaves of *Warburgia* species using the non-polar and moderately polar solvents in combination with longer extraction times at moderately low temperature (Kubo, 1995; Rabe & Van Staden, 2000; Drewes *et al.,* 2001; Khumalo *et al*., 2019). It is therefore not surprising that the initial extraction conditions of 50% aqueous ethanol ($v.v^{-1}$) excluded the extraction of *W. salutaris* drimane sesquiterpenes. The potential for flavour applications in the food and beverage industry could be far greater when these pungent metabolites are extracted, together with the pungent alkaloid, piperanine.

With the growing demand for natural flavours, colours and preservatives, extracts of *Warburgia salutaris* leaves with enhanced extraction yields of piperanine and pungent sesquiterpenoids, potentially hold great value as a novel flavour source with potential functionality (Rabe & Van Staden, 2000; Egebjerg *et al.*, 2009; Leonard & Viljoen, 2015; Mgbeahuruike *et al.,* 2017; Khumalo *et al.*, 2019; Nazir *et al.*, 2019; Piplani *et al*., 2019).

Recommendations for future research would be to investigate the bioactive concentration of piperanine, in addition to sensory evaluation of the pungency at these concentrations to determine whether the flavour would be desirable and/or attainable in a food or beverage product. Similarly, targeted extractions of the bioactive sesquiterpenes in *W. salutaris* leaf powder as well as sensory research of these extracts could be potentially valuable to the flavour industry.

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